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Nuclear hypocrisy in India

'NOTHING will better serve the interests of those who are hostile to us than for us to lose our sense of perspective and to undertake measures which would undermine the basic progress of the country. We believe that to be militarily strong, it is equally important to be economically and industrially strong'. Thus Mrs Gandhi in 1968 in declaring that India would develop her atomic energy programme exclusively for peaceful purposes. In the strictest sense, the recent detonation by India of a nuclear device does not violate any of the carefully worded statements of the past. But if the Indian government believes it can explain away its possession of nuclear devices by careful wording of statements, and can justify a peaceful-purposes programme on economic grounds it is guilty of wicked hypocrisy. Whatever the celebrations in India, scientists there should know that they gain no increased respect from scientists in other countries for producing a nuclear explosive, rather they lose it for being party to such a transparent deception.

The explosion was detonated in the Rajasthan desert—a mere 90 miles from the Pakistan border—and had a yield of 10 or so kilotons. It was fired sufficiently close to the surface (perhaps no deeper than 100 metres) so that it produced a large crater, the purpose of which, apparently, is water storage. There are no immediate reports of radioactive leakage so the experiment must have been done with some delicacy. There have been several cratering shots both in the United States and the Soviet Union and the literature on the subject is relatively open.

Can a nuclear explosives programme be purely peaceful? There is nothing in the science and technology which differentiates between peaceful and military devices, so the distinction can only be in the mind of the firer. Whatever Mrs Gandhi may say about her own intentions she is not going to dismantle the devices when she resigns from office, and her successor could well have much more aggressive intentions. Even in Mrs Gandhi's term of office a political situation could easily arise in which the Government 'with the greatest reluctance' was forced to consider the option of commandeering peaceful devices for warlike purposes. Thus India possesses all the psychological and physical ingredients for a nuclear deterrent and deceives none of its neighbours by talking about making holes and mining copper. The means of delivery are also there in the form of Canberra bombers; despite recent pronouncements India does not need missiles to become a military nuclear power to be reckoned with.

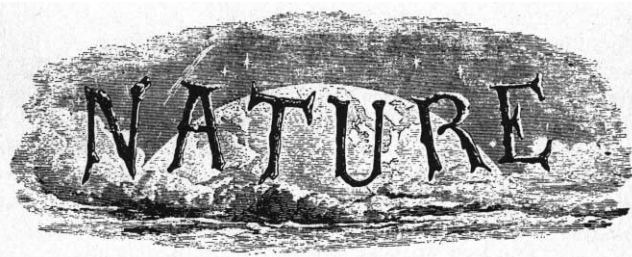
Can India afford it? It costs several hundred million pounds to develop nuclear devices. The cost of the hole in the desert and of any future project could if produced in more conventional ways hardly amount to this—and could employ and feed large numbers of people. If the programme is to be justified on its peaceful benefits, and is not to 'undermine the basic progress of the country' the government has a lot of explaining to do to its people, particularly the hungry ones. For a country with a GNP per capita of £40 there are surely more effective economic ventures than this.

What will be the impact on other potentially nuclear

countries? The test is bound to trigger concern in Pakistan, but a more important question is the reaction of many other countries with nothing to fear from India. There are several abstainers from the Non-Proliferation Treaty who are bound to feel increasing pressure as a result of India's action, notably Israel and South Africa. India has compounded the seriousness of the situation by showing not only that even a poor country can make nuclear devices but also that there is a way in which they can be given some sort of flimsy justification in terms of peaceful uses.

Two golden opportunities have been lost. If India had been prepared to press her need for peaceful nuclear explosives in international circles, it is not out of the question that she could have been responsible for establishing some sort of agency which would have removed peaceful explosions from their present national basis. And if the present nuclear countries had busied themselves more over nuclear arms control in the past 5 years, they would have a better moral platform from which they could harangue proliferators.

100 years ago



AN excellent device has been forwarded to us for use in field-club excursions. It is designed to promote an interest in common flowers, and can of course be varied and worked without a prize. It consists of a large envelope, with a description, but not the name, of a plant, and directions as to what ought to be done with the plant when found. The particular envelope, forwarded to us by Mr. Higgins of the Liverpool Naturalist's Club, contains the following on its back :—

EXTRA PRIZE.

DESCRIPTION OF PLANT.

Leaves opposite, Sessile, Lanceolate, Acuminate.
Sepals 5, half as long as the 5 deeply-cleft Petals.
Stamens 10, Styles 3, height about 12 in.

Members finding a plant answering to this description should take it to the President or Botanical Referee, with their name signed at the foot of this slip. When correct the slips will be initialed and handed to the Secretary. The finder should be prepared to answer questions on the description; but the name of the plant will not be officially announced till after tea.

A Prize or Prizes will be awarded at the end of the Season to those most successful.

Signed, _____

From *Nature*, 10, 95, June 4, 1874.



European space looks forward to the 1980s

*This survey is by Adam Dattner,
Assistant Director of ESRO.*

THE new unified European Space Agency (ESA) is scheduled to come into being next month. It will carry on with the execution of various space programmes which have already been decided on and initiated in the framework of ESRO (the European Space Research Organisation).

The accomplishments of ESRO so far have been mainly in the field of scientific space missions, and the seven scientific satellites launched between 1968 and 1972 have all been remarkably successful. This record of technical success has resulted in a number of scientific achievements, mainly in magnetospheric physics and astronomy but also in solar physics and aeronomy. In particular, remarkable ultraviolet observations of stars have been made with high spectral resolution, as well as one of the first attempts at measuring celestial γ rays.

Four of the satellites launched are still in orbit and operating successfully. In particular two highly eccentric orbit satellites, HEOS A1 and HEOS A2, which measure the properties of magnetospheric plasma, solar wind and the interplanetary magnetic field out to some 30 Earth radii. ESRO IV, in a near-Earth orbit, measures a variety of charged and neutral particles, their temperature, density and composition—particles typical of the upper ionosphere as well as those of solar origin. TDIA, apart from its detectors for solar, X and γ rays, as well as celestial X and γ rays and primary cosmic rays, has two ultraviolet telescopes aboard for high resolution stellar spectroscopy and celestial scanning.

The development programme at present being carried out and the one planned for the near future include sophisticated missions which will pursue the exploration of the Earth's environment with investigation of the magnetosphere, using the GEOS spacecraft (a geostationary satellite to be launched in 1976) and IME (International Magnetospheric Explorer to be launched in 1977 as a part of a three-spacecraft collaborative mission with NASA).

Both will measure typical magnetospheric parameters but GEOS is characterised by its extremely high data flow and advanced experimental

techniques; IME together with the other NASA spacecraft will provide a tool for a synoptic study of the magnetosphere where spatial variation of local parameters is separated from the temporal one.

In the field of astronomy the European community of astronomers is to participate in the joint United Kingdom/NASA IUE (International Ultraviolet Explorer) geostationary mission which is to carry a high resolution ultraviolet telescope and spectrometer to which the European scientists will have access through a Data Acquisition ground facility in Spain. Also in astronomy, but this time not ultraviolet, are the ESRO missions COS-B, carrying a γ -ray telescope and EXOSAT carrying an X-ray telescope; the latter mission will no doubt bring X-ray astronomy a step further, thanks to the excellent angular resolution that can be obtained by using lunar occultation.

Looking to the still more distant future, the scientists are now studying missions of various kinds and one of the dilemmas they will eventually face is brought about by the development of Spacelab. This facility will have features distinctly different from those the scientists have been used to in

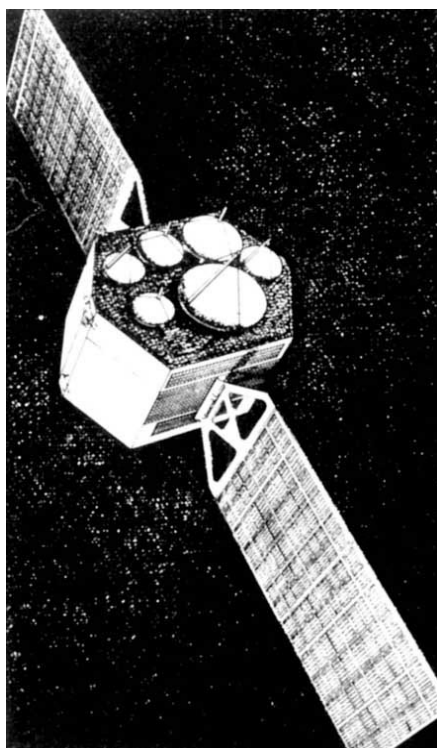
previous missions: the substantially larger payload capability will enable them to accommodate large instruments and crew but the orbital limitations will make *in situ* magnetospheric measurements beyond, say, 1,000 km altitude impossible. Furthermore the duration of missions will be limited but repetition of them will probably be quite frequent, and man's presence aboard will make the missions more flexible. The requirement for long period monitoring will no doubt remain and automatic satellites in many fields of research will complement the measurements on board Spacelab.

Astronomy remains one of the main areas of interest with continuing investigations in the ultraviolet, X-ray and γ -ray regions of the spectrum and extensions into the infrared.

Future missions being studied aim at infrared observations using either automatic satellites for sky surveys, or a larger Spacelab-borne telescope with good pointing accuracy for measurement of emission and absorption lines. Furthermore advanced missions in ultraviolet and X-ray celestial spectroscopy are envisaged, mainly using Spacelab. Solar physics with high spectral and spatial resolution remains at the centre of interest, using both Spacelab-borne telescopes and automatic satellites, depending whether the mission objective is a detailed, high resolution study of phenomena that cannot be observed from the ground or continuous monitoring of the solar disk. Also a Spacelab-borne facility for magnetospheric, ionospheric and atmospheric research is being considered, as well as automatic satellites for measurements of fields and particles out of the ecliptic plane or far out in the Solar System.

Since 1971 ESRO activities have also been oriented towards space applications on which heavy emphasis has been placed. The Meteosat programme is to be Europe's contribution to the space element of the Global Atmospheric Research Project (GARP) which will be carried out in 1976 and 1977 prior to establishing a worldwide system to be part of the World Weather Watch (WWW). The geostationary Meteosat project will carry a radiometer to provide good resolution photographs of the Earth in the visible and infrared. As from late 1973 the project has been in the development stage and the satellite will be launched at the end of 1976.

OTS: orbital test satellite



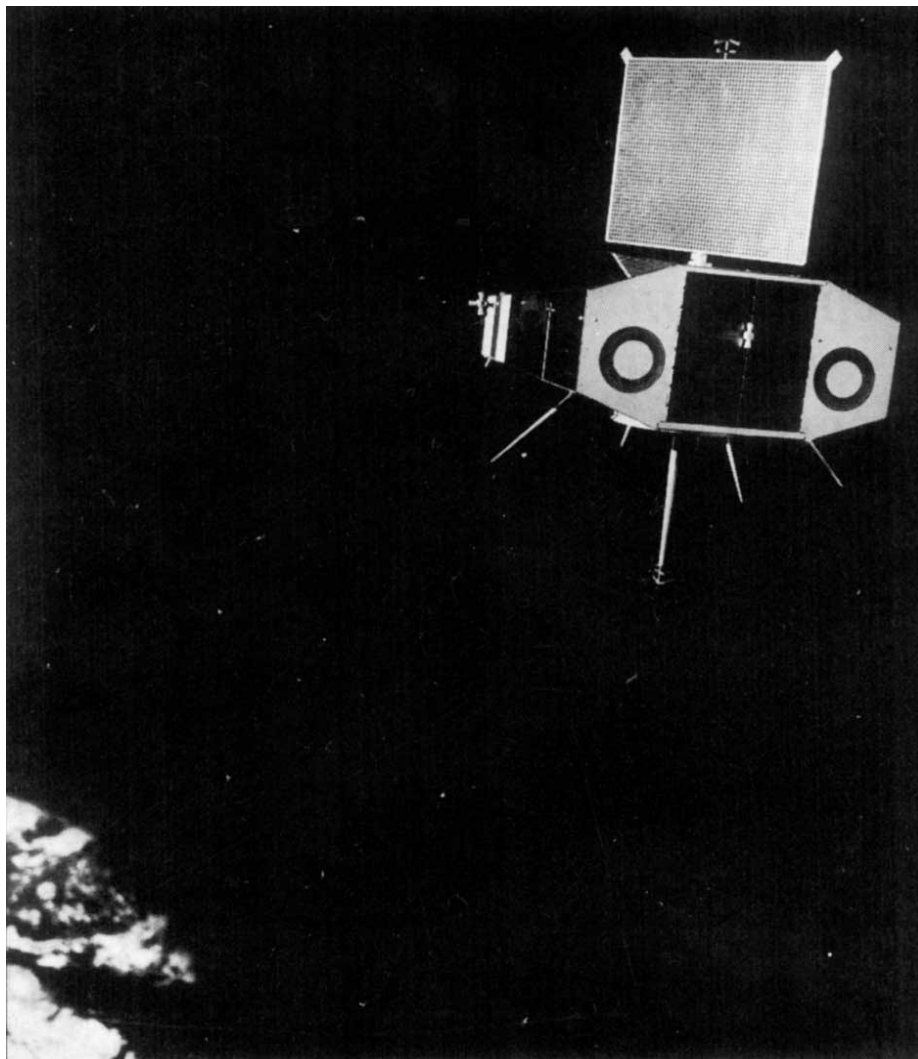
EXOSAT : highly eccentric orbit satellite to be launched in 1979, carrying an X-ray telescope in the 0.1–20 keV range, using lunar occultations for high resolution determination of X-ray sources.

A geostationary satellite, the Orbital Test Satellite (OTS), is under development for telecommunications, experiments and pre-operational use, its beam covering western Europe and the Mediterranean. It is to be a precursor of an operational communications satellite system to satisfy the needs of the European Postal and Telecommunications Services in the early 1980s, some four years after the launching of OTS in early 1977.

The increased air-traffic density over nonpopulated areas and the inadequate high frequency communications now used for aircraft over such areas give rise to an obvious application of satellites for air traffic control. Aerosat a collaborative venture of ESRO and the United States aims at establishing a pre-operational system of geostationary satellites, in the first instance over the Atlantic.

Marots, the latest newcomer to the family of ESRO's applications missions, is another example of space technology's potential for communications. The spacecraft technology being developed for OTS is perfectly suitable for maritime communications. The beam of the geostationary satellite will cover a large part of the hemisphere centred on the Greenwich meridian. The spacecraft is going to be launched in mid-1977, about six months after the launching of OTS.

Two other new projects recently included in the ESRO programme are Spacelab and Ariane. The United States shuttle programme, now going ahead at full speed, will permit the placing into orbits of several hundred kilometres altitude of an aircraft-like vehicle roughly the size of the present DC9. The vehicle is recovered at the end of each mission as well as many elements of the associated boosters used for launching. The 'Sortie Module', or as it is now called 'Spacelab' (a fully equipped modular laboratory placed into the vehicle) is being developed by ESRO as part of a collaborative programme with NASA. Spacelab can consist of a combination of modular elements, assembled to either a cylindrical container some 6.5 m long and 4.5 m in diameter, or to other configurations consisting of a smaller Spacelab capsule and external pallets on which experimental equipment, such as astronomical telescopes and other sensors, can be mounted. The large volume available for experiments operated and main-



tained by humans, the large weight-carrying capability and the power available—all combined with a shirt-sleeve environment, with data processing and other facilities aboard—destine Spacelab to become by early 1980 the most flexible tool for space experimentation and space operations ever built.

The missing element in Europe's space capability has always been heavy launchers. The Ariane launcher (originally the French L3S, now Europeanised) is to fill the gap. A three-stage vehicle, using high energy propulsion in its third stage, will be able to place 750 kg into geostationary orbit when launched from the Kourou range in French Guiana. Four test launches are foreseen between the beginning of 1979 and mid-1980.

The almost simultaneous start of all the programmes described above, and thus the concentration of launches in 1976 and 1977, will put a strain in the next two to three years on Europe's resources devoted to space. In spite of this the interest for other space programmes remains, and several other applications are being actively studied. Data transmission from a satellite

seems to offer interesting advantages and television broadcasting seems to be another obvious candidate for space application. The United States' experience in experimental surveys of Earth resources indicates that this application may be one of the more important ones in the future. ESRO is also studying the possibility of such missions.

Recently a possibility of determining, with extremely high accuracy, the position of radio emitters on the ground, on the sea or in the air (using orbiting satellites) attracted attention and a French proposal to undertake a project called Geole is being discussed. The proposal is to use such techniques for cartography, glaciology and also for mineral prospecting and exploitation.

This list is by no means exhaustive and it has been the experience of all those involved in space research that new applications are constantly being found. Space research is only 17 years old—the first Earth satellite was in 1957. It is only in the 1980s that humanity will fully make use of this new and promising field.

international news

Mr. Varley reports on North Sea energy

Eleanor Lawrence

ALL set fair on the energy front was the message that the Secretary of State for Energy, Mr Eric Varley, delivered in his annual report to Parliament on the state of Britain's oil and gas reserves (*Production and reserves of oil and gas in the United Kingdom: a report to Parliament by the Secretary of State for Energy*, HMSO, 32p).

Assuming the forecasts are correct, Britain should be self sufficient in oil by 1980. At present, consumption of oil in the United Kingdom runs at around 100 million tons a year and is expected to rise in the next 10 years. Oil production could well reach 100–140 million tons a year by 1980 and be sustained at this rate throughout the decade, says the report. Although present proven commercial reserves could not keep this sort of production up, it is confidently expected that further commercial finds will be made in areas already licensed in the North Sea, the Celtic Sea and areas that will be explored when licences are granted. Indeed, as Mr Varley was introducing the report Occidental Oil announced a new find, the Claymore Field near its Piper Field off the Orkneys.

But the estimates for the first oil ashore in 1975 have been downgraded from 25 million tons (last year's estimate) to 5 million tons. This setback is due to delays in obtaining and installing pipeline and production equipment as a result of materials and labour problems.

On the thorny problem of finding building sites for the massive production platforms, Mr Varley said that he had no plans in hand to bring in legislation. At present the industry is not unduly worried about a shortage of platform building sites, but Mr Varley warned that his department would not hesitate to ask for legislation to speed up present planning procedures if it became necessary.

The first oil ashore is expected to come from the big Forties field con-

trolled by BP. This is now expected to start production in 1975 after delays in installing steel production platforms that were supposed to have been in position in 1973.

Estimated reserves of oil in the North Sea now total 895 million tons from proven sources with a possible total of 2,950 million tons when all possible sources already discovered are taken into account. These figures compare very favourably with last year's estimate of 70–100 million tons with reserves of 800–1,300 million tons to be proved to sustain the forecast production level. The report stresses the difficulties in giving accurate estimates of production levels and reserves, especially as no oil has actually come ashore yet. More realistic figures should be available when the fields have been operating for a few years.

In contrast to oil, the North Sea gas industry has now been in operation for several years. Reserves remaining are estimated to be sufficient to provide between 5,000 and 6,000 million cubic feet a day, about double last year's consumption, in the late 1970s. This rate of production could be sustained with existing reserves until the 1980s but unless new reserves are found production will then slowly decline. Exploration for new reserves is now being concentrated in parts of the North Sea other than the heavily developed area off the south-east coast of England and the northern basin, which includes the Frigg field, and seems extremely promising.

Commenting on the report, Mr Varley said that Britain was better placed for energy in the medium term than many other industrial countries. In the 1980s some crude oil of the heavier grades would have to be imported for certain purposes such as the manufacture of lubricants but otherwise Britain would probably be physically self sufficient for oil. When asked if he planned any further controls to prevent oil companies sending the oil abroad, he said that present regulations ensured that all oil from the North Sea would be landed in Britain and that present export licensing arrangements would be sufficient controls. Self sufficiency in oil means, however, that a great deal of the oil from the North Sea will have to end up as domestic and industrial fuel given current consumption in the United Kingdom. Some people are of the opinion that the high quality North Sea oil is too

valuable to burn and that Britain would get better value from her oil if it were channelled into the manufacture of petrochemicals for export.

Although he regards the turn of Britain's energy fortune as very encouraging, Mr Varley tempered the general air of euphoria with a warning that the oil and gas reserves will not last for ever and that the best possible use must be made of them. To these ends he committed his department to investigating ways of conserving energy although he conceded that this would be extremely difficult to do for the domestic consumer.

Test ban talks draw liberal fire

Colin Norman, Washington

THE recent announcement that the United States and the Soviet Union are trying to negotiate an agreement which will limit the size of underground nuclear explosions (but not ban them entirely) has drawn fire from the Federation of American Scientists (FAS), an intellectually heavyweight organisation which lobbies chiefly for arms control, and from a handful of liberal senators. The federation has warned that such a treaty would probably have little effect on the development of new nuclear weapons and that it would have no effect on nuclear proliferation.

The fact that a so-called threshold treaty is under discussion surfaced earlier this month, before India joined the nuclear club, and it is generally believed that some form of agreement will be ready for President Nixon to sign when he travels to Moscow for a summit meeting at the end of June. It is cynically being viewed in Washington as a device which will allow Nixon to maintain his self-proclaimed image as a peacemaker even though he will be unlikely to bring back from Moscow any major new arms limitation agreement resulting from the strategic arms limitation talks.

A threshold test ban treaty is likely to set a limit on the magnitude of seismic signals which can be sent out by an underground nuclear explosion. In other words, it will enable both the United States and the Soviet Union to carry on testing small nuclear devices provided that they do not send out seismic signals greater than whatever threshold is negotiated.

An FAS statement, released at a press conference last week and endorsed by five former US disarmament negotiators, argued that unless the threshold is set at a very low level it is unlikely that the number of tests will be reduced below their present level. This is particularly true since two major weapons programmes—the development in the United States of small, low yield tactical nuclear explosives and the building in the USSR of new and improved MIRV warheads—require tests which will probably be below the threshold.

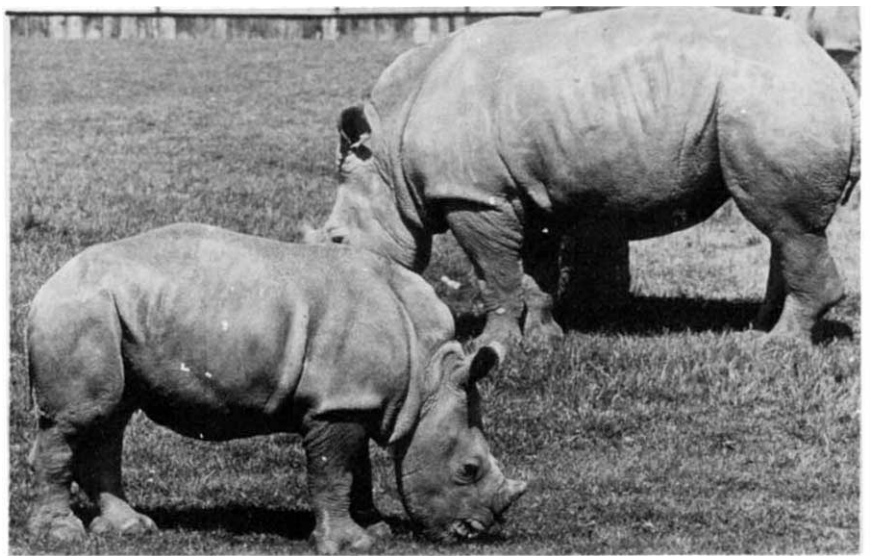
As Dr James Leonard, the US ambassador to the Geneva disarmament negotiations between 1969 and 1971 and now a Vice President of the United Nations Association, put it at the press conference, “A threshold treaty is a reflection of the philosophy that the United States needs to keep testing”.

The FAS also emphasised the difficulties that would be involved in making sure that a threshold treaty is not violated. Since the seismic signals sent out by an underground explosion would not necessarily be of the same magnitude at all instrumentation stations, an explosion which in fact is below the threshold could register as being above it. “Thus, not only will the negotiation of an international verification system be extremely difficult”, the statement says, “but even with the best of intentions suspicions of cheating are likely to arise”.

Another serious reservation expressed by the FAS is that a threshold treaty would do little to halt the spread of nuclear weapons—a topic which has suddenly been thrust into the foreground with the announcement of India's first successful nuclear test on May 18. Leonard, for example, suggested that such a treaty “would be seen by others in the world not as a serious disarmament measure but as a sham—a fraud by the United States to protect its right to test”. He added that “it would not only be seen that way, it would be used that way”.

Since many non-nuclear nations have refused to sign the nuclear non-proliferation treaty unless the super powers limit their development of nuclear weapons, a threshold treaty which allows continued testing would do little to improve the prospects for halting proliferation. Similar sentiments were also expressed last week by Senator Edward M. Kennedy, who warned that a threshold treaty “would be seen by many nations . . . as continued hypocrisy by the super powers”.

Kennedy, who has recently returned from a trip to the Soviet Union reports that he is convinced that the time is now ripe to press ahead with negotiations for a comprehensive test



White rhino born at Whipsnade, 1973.

DESPITE fears that the higher entrance charges as a result of the introduction of Value Added Tax would discourage visitors, both London Zoo and Whipsnade had even higher attendances last year and finished 1973 in surplus. For the first time since 1968 more than two million people visited the London Zoo in 1973, spending a total of £1 million. Introducing the Annual Report, the Secretary of the Zoological Society, Lord Zuckerman, said that the high attendance, in the absence of an outstanding animal event or the opening of any major building, reflected the very high standard of ‘normality’ at the Zoo. The cost of the research and educational programmes that the society maintains amounted to more than £0.5 million in 1973. The two research institutes in the society's grounds, the Nuffield Institute of Comparative Medicine and the Wellcome Institute of Physiology cost £189,000 and £49,000, respectively, to run last year and of this the society directly con-

tributed £183,600 from its income from such sources as entrance fees. Throughout the report, the theme of the role of the zoo in conservation is apparent. Births in captivity of rare or endangered animals such as white rhinos, cheetahs and orang-utans are given prominence, and this function of the present-day zoo is picked up in another of the society's publications, the *International Zoological Yearbook*, which for the first time has a special section devoted to the ethical and practical problems of trading in and rearing rare wild animals. Geoffrey Schomberg, the Secretary of the Federation of Zoological Gardens of Great Britain and Ireland, writing in the Yearbook asks: “Are endangered species a proper object of retail trade in any circumstances?” Too many zoos, he says, while paying lip service to the ideals of conservation become fiercely competitive in acquiring rare specimens.

ban. He said that from his “discussions with Soviet leaders, I believe that they would be willing to move forward in this area”.

Backed by 36 co-sponsors, Kennedy last year introduced into the Senate a resolution calling on the United States and the Soviet Union to seek an immediate moratorium on nuclear testing and to begin serious negotiations toward a comprehensive test ban. That resolution will be on the floor of the Senate in the next couple of weeks and if it is passed it will send a clear signal to the Administration. Since the Senate will have to ratify whatever treaty is negotiated, if it insists on a comprehensive treaty by passing Kennedy's resolution intact according to some observers, it could have one of two effects. Either the Administration will try to negotiate a treaty with

as low a threshold as possible, or the hand of those within the Administration who have argued for a comprehensive ban will be strengthened to such an extent that the negotiations will be aimed not at a threshold but at a complete test ban.

One concern voiced both by Kennedy and the FAS is that negotiation of a threshold treaty could forestall completely the achievement of a comprehensive ban. As the FAS statement put it, “We understand very well that the politics of summitry and the politics of impeachment both impel the Administration towards an arms control agreement in June in Moscow but we do not want to see an important element in our national security—the complete test ban—sold out in favour of a much less useful agreement”.

AFTER years of secrecy, evasion and even outright denials, the Department of Defence has at last admitted that it carried out an extensive weather modification programme during the Vietnam War. The operation, which took place between March 1967 and July 1972, was designed to increase rainfall over the Ho Chi Minh Trail and make it difficult for the North Vietnamese to move troops and equipment into South Vietnam along muddy roads and jungle trails.

The Pentagon's admission came during top secret hearings held by a Senate subcommittee, on March 20, a transcript of which was made public last week. The transcript reveals that the rainmaking operations were so highly classified that even some senior Pentagon officials were unaware of them, and that there is now a deep division of opinion within the Department of Defence about their effectiveness.

According to testimony given at the hearings by Lieutenant Colonel Ed Soyster, a total of 2,602 cloud seeding missions were flown over Laos, Cambodia and North and South Vietnam, and 47,409 cannisters of silver and lead iodide were expended during the five-year programme. The objective was to supplement natural rainfall during the normal rainy season, and to extend its length, thereby causing landslides, washing out bridges and generally turning trails into a sea of mud.

Although it has been rumoured and reported in newspapers for about three years that the United States indulged in rainmaking operations during the Vietnam War, the extent of the programme revealed during the Senate hearings has surprised many observers.

The operations resulted from an extensive test carried out in October 1966 in Laos. According to Soyster's testimony, 56 cloud seedings were conducted, with a success rate of more than 85%, and the Commander in Chief of Pacific Operations concluded that rainmaking over the Ho Chi Minh Trail "could be used as a valuable tactical weapon". Operational cloud seeding missions began on March 20 the following year.

An air force base in Thailand was used for the operation which was carried out with weather reconnaissance aircraft. In short, cloud seeding consists of dropping burning photoflash-type cannisters into an updraft in a cloud mass. As the cannisters burn, silver iodide or lead iodide is produced and this acts as a seeding agent, causing drops of moisture to form in the cloud, which eventually fall as rain. The entire military rainmaking operation cost about \$3.6 million a year.

Pentagon admits Vietnam rainmaking

Colin Norman, Washington

Initially, the area chosen for cloud seeding consisted of South-eastern Laos and a small area of North Vietnam, but later in 1967, operations were extended into North Vietnam and a small region of South Vietnam. Finally, in 1972, portions of Cambodia and a larger region of South Vietnam were included.

All cloud seeding activities over North Vietnam were, however, halted on November 1, 1968, the day that President Lyndon Johnson ordered a bombing halt, and they were never reinstated. And the entire operation was ended on July 5, 1972—just two days after the *New York Times* published a lengthy account of the Pentagon's rainmaking activities.

Soyster told the committee that although it was usually possible to seed every cloud within a target area, priority was given to seeding clouds directly over roads, intersections and river crossings, which indicates an accuracy for the operation far greater than many independent observers believe possible.

How successful was the programme? According to Soyster, the Defence Intelligence Agency calculated that "rainfall was increased in limited areas up to 30% above that predicted for the existing conditions". He added that sensor recordings of movement along the Ho Chi Minh Trail "indicated enemy difficulties from heavy rainfall", and said that in April 1971 there were about 9,000 enemy logistic movers in Laos each week, but by the end of June the number had dropped to 900. The greatest decreases in troop movements came during the first week in June, when a typhoon struck the area, and during the third week in June, when the Pentagon's rainmaking activities were at their height.

The Pentagon's admission that it has indulged in weather modification came after persistent attempts by Senator Claiborne Pell, a Rhode Island Democrat, to get at the facts. Pell, who is Chairman of the Subcommittee on Oceans and International Environment of the Senate Foreign Relations Committee, proposed a resolution which the Senate passed last year by a vote of 82 to 10 calling on the United States Government to launch international negotiations aimed at banning weather modification as an instrument of war, and it was his subcommittee which held the top secret hearings on the military rainmaking operations. The transcript

of those hearings was declassified by Secretary of Defence James Schlesinger.

Details of the rainmaking operations were held in strict secrecy, presumably because of the sensitivity of the programme. According to Soyster, individual mission reports were transmitted through special communication channels to the joint chiefs of staff, who made periodic reports to the Secretary of Defence and the White House.

Furthermore, since reports and rumours of the operation appeared in the press, the Pentagon has been more than evasive on the matter. During hearings held by the Senate Foreign Relations Committee in April 1972 for example, then Defence Secretary Melvin Laird was asked whether the military had ever indulged in rainmaking activities. He replied: "We have never engaged in that type of activity over North Vietnam", but in January this year he wrote to the committee to apologise for his mis-statement, saying that he was unaware of the weather modification operations carried out over North Vietnam by the Johnson Administration. He was, of course, fully aware of the rainmaking activities he had authorised over Laos, Cambodia and South Vietnam, but simply evaded any mention of them.

The Pentagon's obsession with the secrecy of the programme was taken to its final extreme in 1972, when it refused even to supply details of the operation to an interagency task force chaired by Herman Pollack, director of the bureau of international scientific affairs in the Department of State, which was trying to develop administration policies on both military and civilian weather modification. Although that study itself was classified, the Pentagon has now admitted that the rainmaking operations were so heavily classified that it refused to turn over any information to Pollack.

Since the skies over South-east Asia were raining high explosives and all manner of unsavoury weapons during the Vietnam war, why did the Department of Defence choose to hide the fact that it was tinkering with the weather? One reason is that the whole area of weather modification is a sensitive one simply because it is a new area of warfare which may eventually open up some frightening possibilities.

As Pell puts it, "We should not open the Pandora's box of harnessing nature, of changing the weather or developing techniques to create typhoons or earthquakes with devastating effects upon foes and neutrals alike. I believe this new kind of weaponry should be eschewed by developed, so-called civilised nations".

Club of Rome associations

David Spurgeon, Ottawa

CANADA is the latest country to have formed a national association inspired by the Club of Rome. The decision was made at a meeting in Toronto early in May, and the venture has the backing of an impressive array of government and business officials.

The association's objects are "to promote study and discussion among all segments of the Canadian public of the nature of world problems and the need to develop new policies, attitudes and courses of action to ensure a stable and viable future for mankind . . ." More specifically, it will attempt to identify Canada's role in the solution of world problems, and the implications for Canada of possible world solutions.

Both the co-founders of the Club of Rome, Dr Aurelio Peccei of Italy and Dr Alexander King, who until recently was with the OECD in Paris, attended the Toronto meeting, which was opened with a welcoming letter from Canada's Prime Minister, Pierre Trudeau. From their accounts it was apparent that the club—largely through the impact of the MIT study it sponsored, *The Limits of Growth*—had made considerable progress in directing attention to world problems since its inception in 1968.

One reason for thinking so, as suggested by Dr Peccei, is the establishment of a series of United Nations Conferences for discussion of world problems such as population, food, energy and materials. Another is the organisation in many nations of groups like the Club of Rome.

As described by Dr King, these included a whole diversity of approaches. The first was set up in Holland, which according to King is not surprising, because of its twin problems of dense population and heavy industrialisation. The Dutch association is a group of private people gathered in a nonparty organisation.

The second—also not surprisingly, for the same reasons—was organised in Japan, on a different basis, backed by and formed as a committee of the techno-economic society. Groups in other countries include some not connected with the Club of Rome but interested in similar problems (for example, Great Britain and Austria), and luncheon clubs and groups of engineers or teachers in France, Denmark, Belgium and Sweden.

Finland has formed a more formal study group to determine what the role should be of a small, progressive country in the modern world; Switzerland, with four Club members, invited four government members to discuss



BENEATH the elegant tapestries of the Schlosshotel, Kronberg, Germany a small group of biologists, philosophers and psychiatrists gathered recently to constitute the Boehringer Ingelheim Symposium on the Creative Process in Science and Medicine. Pictured above, listening to Sir John Eccles testify are, in the front row, Sir Hans Krebs, Jacques Monod, Manfred Eigen, Desmond Morris and Sir Karl Popper. What leads to some scientists being creative way above the average, and does the creativity of scientists bear any resemblance to that of artists and composers? Participants were decidedly coy about speaking other than in the abstract, despite Krebs's personal example of trying to piece together how he came to produce

new ideas. All seemed agreed that breaking out from conventional thinking was a major ingredient in creativity—the agreement was so complete that it must qualify as conventional thinking and therefore . . . Monod discussed 'subjective simulation'—a process almost of putting yourself in the position of the electron or whatever that you are thinking about. The debt of many participants to Popper was clearly great—an interesting contrast with the world of physical science where one's impression is that there is less understanding of what he is about. Desmond Morris had probably the nicest story to tell—A journalist once asked Picasso: What is creativity? 'I don't know, and if I did I wouldn't tell you.'

Club of Rome thinking and found them receptive to the points where they decided that both the traditional political organisation and neutrality of the country was obsolete.

But Dr King found some of the keenest interest in an unexpected place: South Africa. I was in South Africa recently, he said, "and, much to my surprise, found the Club of Rome better known in that country than in any other." Some citizens there have formed an independent information group who just meet and talk.

The Canadian Association for the Club of Rome has applied for a charter as a corporation without share

capital. Its first directors include the president of the pulp and paper industry's research institute, Dr Pierre R. Gendron; Senator Maurice Lamontagne, whose senate committee carried out one of the most comprehensive studies of national science policy yet made; a retired oil company executive, Mr Ronald Ritchie; the president of the Ontario Research Foundation, Dr William Stadelman; the former Chief Science Adviser to the Federal Cabinet, now Dean of Applied Sciences at Queen's University, Dr Robert J. Uffen; and a senior civil servant with many years experience in Canadian Science policy, Dr J. Rennie Whitehead.

correspondence

Canadian science

SIR,—In your issue of March 15, David Spurgeon offered his own interpretation of recently announced changes in the structure of federal scientific activities. After reading his article, I cannot avoid feeling that the author has done a disservice to Canadian scientists.

Spurgeon makes the statement that the press releases of the Ministry of State for Science and Technology served only to confuse in an attempt to provide more detail about the rather general statements made in the throne speech. I submit that he is guilty of that particular offence.

"The question in the minds of some", wrote Spurgeon, "was whether the ministry was finally becoming . . . an all-powerful, monolithic instrument of power in Canadian science policy". He went on to say that a quick sampling of reaction in the science community indicated that the ministry's releases had indeed given this impression. The sample must have been a very quick one because, following the release of the information kit, a survey of Canadian scientific opinion published in the daily press indicated that most of those surveyed were not prepared to condemn the changes out of hand but instead would wait for specific actions before passing judgment.

Spurgeon cites, as perhaps the most confusing element of the announcement, the failure of the ministry to mention anything about possible new reporting relationships for the granting councils. Surely Spurgeon is hung up on the administrative considerations he so vigorously condemns. Reporting relationships are simply provisions for accountability to Parliament. What is important to every scientist are factors such as peer assessment of research proposals, the allocation of funds to the granting councils and the input of the granting councils to the Inter-Council Coordinating Committee. All these factors were clearly dealt with in the ministry press releases. The reporting channels for the existing granting councils have not been altered.

Finally, Spurgeon questions the separation of the granting function of the National Research Council of Canada from the laboratory function. Such a separation, he says, will destroy a vital link between government research and industry. The changes proposed for the NRC affect only the

university granting mechanisms. The traditional liaison between the NRC and industry will continue.

Yours faithfully,
AURELE BEAULNES

Ministry of State, Science and
Technology,
Ottawa

Journals jungle

SIR,—I am writing in reference to the article "Science Journals in a Prices Jungle," which appeared in the *Nature* dated February 15, 1974. That article discussed price rises of a variety of journals and listed "a record-breaking increase" for *International Pharmaceutical Abstracts* of "261%". I must respond to this kind of reporting since not all the information was included; this makes the information that does appear misleading.

Prior to January, 1973, *IPA* had two subscription prices—an institutional subscription price of \$100.00 a year and a subscription price to individuals of \$40.00 a year. A two-subscription price system was difficult to administer since many people who should have paid \$100.00 were paying only \$40.00, and so on. We were faced with having to increase both rates since we were losing money in the situation that existed at that time. The expense of publishing *IPA* was increasing each year and that just could not be balanced by income at existing subscription rates. After a thorough study of our subscribers, we decided to change to one institutional subscription price of \$150.00 a year. The increase was from \$100.00 to \$150.00 and not quite as record breaking as the article implied. We felt, however, that we could not ignore the individual completely and therefore decided to offer an additional copy subscription rate of \$30.00 a year (if one subscription exists at the \$150.00 rate, as many additional copies as desired can be obtained at the \$30.00 rate).

Other facts relating to the change are that we improved and expanded the services offered. All our promotional pieces and literature at that time mentioned that we would be providing greater coverage, most abstracts, and that *IPA* would be more current. This in fact has happened, and in 1973 we published 13% more abstracts than we did in 1972 and *IPA* was on time. We did

follow through on our promises of additional services for the change in subscription price—it was not just a price change for the same service. One other fact that seems to have been completely overlooked is that *IPA* is still one of the lower priced secondary publications. Most abstracting-indexing publications are far more than \$150.00 a year. Possibly our real problem is that we began at too low a subscription price for *IPA* when it began.

Yours faithfully,

DWIGHT R. TOUSIGNAUT
American Society of Hospital
Pharmacists,
4630 Montgomery Avenue,
Washington DC 20014

Kidney transplants

SIR,—Why publish such a rank piece of scientific journalism as "Transplants: the failing machinery" (April 19, 1974)? The subject has always been an emotive one, but this is not an excuse for the lack of reason displayed and the dramatic aura with which the article is surrounded. To use comparisons between kidneys received from and contributed to the national donor pool as an indication of "reluctance . . . to share out kidneys which are not suitable for their own patients" is nonsense unless some measure of the kidneys potentially available within an institution by comparison with the size of its recipient pool is also given. Both St Mary's and Hammersmith are hospitals with a large transplant programme in areas where irremediable cranial trauma is rare. Further St Mary's does not have a neurosurgical service so that we see few patients with intracranial vascular accidents and such patients as we have with progressive primary intracranial malignant disease usually go elsewhere before they die. So we shall always be debtors rather than creditors.

Is there unequivocal evidence that "lack of understanding and cooperation between doctors . . . is largely to blame"? Of course, being human, we are too lazy, too hidebound and too hardworked always to do the right thing in complicated situations, but I do not know of data to suggest that a large number of kidneys are being lost through medical indifference.

Yours faithfully,

H. A. F. DUDLEY
St Mary's Hospital,
London W2

news and views

Super-rotation of the upper atmosphere

OBSERVATIONS of small changes in the orbital inclinations of artificial satellites have shown that the Earth's upper atmosphere (at altitudes of 150–400 km) is rotating about 20–30% faster than the Earth itself. This phenomena has become known as super-rotation. Even though it was discovered by King-Hele in the early 1960s, the cause of this 100 m s^{-1} west to east wind is still a subject of considerable debate. The change in inclination because of super-rotation is only about 0.1° during the satellite lifetime, and so spatial variations and short term effects are very difficult to detect. No clear evidence has been found of year to year variations, such as might be caused by the 11-yr-periodicity in solar activity, but over daily periods the wind seems to maximise between 2100 and 2400 local time.

Possible causes of super-rotation are:

- Horizontal pressure gradients may be set up in the atmosphere as a result of thermospheric heating by solar ionising radiation, and bombardment by energetic particles. The resulting global pressure distribution produces geostrophic and cross-isobaric winds which are probably controlled by ion drag (the motion of the F region ions borne along by the neutral winds is strongly effected by the geomagnetic field). A problem with this theory is that heating of the auroral zone, which takes place during magnetic disturbances, can reverse the mean meridional pressure gradient. This would cause a drastic change in the super-rotation, a change which has not been observed.
- Super-rotation might be caused by ionospheric or magnetospheric motions which couple with the neutral atmosphere by collision processes. Meridional electric fields can move F region ions quite efficiently even in the presence of the geomagnetic field. An average field directed towards the equator could produce the super-rotation, and these fields have been observed in high northern latitudes during negative magnetic bays. The effect should, however, be enhanced during magnetic disturbances. This is contrary to observations.
- The moving flame effect. A heat source acting on a fluid produces convective motion. If the heat source moves (as, for example, the Sun does in a westerly direction with respect to the Earth's atmosphere) the convective cells tilt, producing horizontal and vertical motions which interact to produce a mean horizontal flow. The flow rate is a function of the frequency of the heating function, and of the ratio of the kinematic viscosity and thermal diffusivity of the fluid. Permissible values of such parameters in the case of the Earth's atmosphere unfortunately yield inappreciable amounts of super-rotation; a situation which compares unfavourably with the case of Venus where the upper atmosphere rotates 50–60 times faster than the planet mainly because the heating function has a period of 240 days (the Venusian surface rotation period) as opposed to 1 day on Earth.
- Atmospheric waves may impart momentum to the F region, in which they are dissipated. If transmission efficiency depends on the direction of wave propagation, a net motion can be produced. As wave velocity is an inverse

function of density, an imperceptible mean velocity at low levels can produce the observed thermospheric velocity.

- The Scott effect may produce super-rotation: a small brass cylinder suspended in a cylindrical vessel containing a low pressure gas, experiences a torque when a vertical magnetic field is applied, and if a temperature gradient is set up between the cylinder and the vessel. This effect might act on the atmosphere.

At present it seems that none of these theories can explain the phenomena of super-rotation completely. A sixth theory has now been put forward by Ved Mitra (Physics Department, MNR Engineering College, Allahabad, India) in a recent edition of *Planetary and Space Science* (22, 559; 1974). In this case, super-rotation is caused by meteoroid deposition. The meteoroids, small interplanetary dust particles which mainly originate from decaying comets, and which move in elliptical orbits around the Sun, carry, on average, a greater amount of angular momentum than that corresponding to the Earth's orbit. When they interact with the Earth's atmosphere the angular momentum must be conserved and therefore the excess orbital angular momentum of the meteoroids becomes manifest as extra spin angular momentum in the atmospheric layers in which they are arrested.

Mitra assumes firstly that the majority of the mass deposited in the atmosphere is in the form of particles with masses 10^{-6} – 10^{-3} g. These meteoroids are assumed to have orbits similar to those of the larger meteors which were recorded photographically by super-Schmidt cameras during the Harvard photographic meteor project. The orbits are, in the main, within Jupiter's orbit, with low inclinations, and high eccentricities and direct motion. When these particles collide with the Earth's atmosphere they cause it to 'spin up'.

For example, particles coming from behind the Earth will be accelerated by gravitational attraction, and this increase in velocity will cause them to move away from the Sun and collide with the Earth from outside, giving it a prograde spin. Particles in front of the Earth, in direct orbits, will lose velocity because of gravitational attraction, strike the Earth on the Sunward side and again impart prograde spin. This increase in spin during gravitational accretion is exactly the same process propounded by Alfvén for the origin of planetary rotation in the Solar System.

Mitra calculates the orbital angular momentum per unit mass for the incident meteoroids, and by comparing this with the angular momentum of the Earth finds the influx required to produce the observed super-rotation of 100 m s^{-1} in the atmospheric layers above 150 km. The answer comes to 34 tons a day, a value in excellent agreement with that of 43.5 tons a day obtained by Whipple (*Smithson. Astrophys. Inst. Prepr.*, No. 239; 1967), who, however, considered the Earth as a nongravitating sphere; 55 tons a day by Dohnanyi (*Icarus*, 17, 1; 1972) who used results of satellite-borne penetration experiments, and ground-based visual and radar observations; and 48 tons a day obtained by Zook *et al.* (*Planet. Space Sci.*, 18, 953; 1970) obtained from observations of micrometeoroid impact on the window of Gemini spacecraft.

Unfortunately, there are two problems with Mitra's argument. The first is minor and concerns the size of

the incident particles. Recent work (for example, Hughes, *Space Res.*, **14**, 789; 1974) indicates that about 95% of the mass incident on the Earth is in the form of particles of mass 10^{-9} – 10^{-5} g which have very low geocentric velocities and different orbits from those assumed by Mitra.

The second point concerns Mitra's assumption that "the effective deceleration of the velocity initially possessed by a meteoroid takes place in the 150–400 km region". This is contrary to visual and radar observations of the more massive meteoroids ($> 10^{-5}$ g) which have maximum ablation at heights of about 90 km. Either it must be assumed that the super-rotation is produced by the minor amount of ablation and momentum transfer that occurs at heights above 150 km, in which case Mitra's 34 tons a day influx must be increased accordingly, or a process of momentum transfer from the lower layers where the ablation occurs to the regions of super-rotation must be invoked; similar to that propounded for the transfer of atmospheric wave energy.

It seems therefore that the cause of super-rotation still remains a mystery and that the Earth's spinning upper atmosphere is still the happy hunting ground for new theories.

DAVID W. HUGHES

The collagen triple helix

JAMES WATSON, in his Nobel address on the work which led to the Watson–Crick model for DNA, spoke of his fear that this structure might have turned out to be "dull and uninteresting, like collagen". In the event, collagen has turned out to be a most interesting protein which has occupied the attention of workers from a wide range of disciplines.

Recently, attention has been focussed on the intracellular process by which the three polypeptide subunits (α chains) of monomeric collagen form its characteristic triple-stranded helix. The very slow and incomplete *in vitro* renaturation of α chains derived from extra-cellular collagen led to the proposal by Speakman (*Nature*, **229**, 241; 1971) that the α chains as initially synthesised might have peptide extensions. It was postulated that these extensions or 'registration peptides' might serve to bring the three α chains into a conformation which would allow rapid formation of the triple helix. Subsequently much evidence has accumulated to indicate the existence of a precursor of this type (called procollagen) during the biosynthesis of collagen in a wide variety of tissues, and some of the properties of procollagen have been defined (reviewed by Schofield and Prockop, *Clin. Orthopaed. Rel. Res.*, **97**, 175; 1973). Initially considerable controversy existed about the size of these extensions and their amino acid composition, but there now seems to be general agreement that type I and type II procollagens comprise polypeptides of 120,000–125,000 daltons. Moreover, it seems that all pro- α chains contain cysteine, an amino acid not usually found in extracellular interstitial collagen, with the possible exception of a newly discovered form of collagen (type III) recently isolated from several human tissues (Chung and Miller, *Science*, **183**, 1200; 1974).

The demonstration of the presence of cysteine in procollagen suggested that disulphide bonding occurs between the pro- α chains and several groups have now reported that procollagen is secreted as a triple-stranded disulphide-stabilised molecule of molecular weight of about 360,000 daltons. Similar observations have been reported by Monson and Bornstein (*Proc. natn. Acad. Sci. U.S.A.*, **70**,

3521; 1973) who also demonstrated that the procedures used previously in their laboratory for the extraction of procollagen from cranial bones resulted in partial cleavage of the extension peptides by endogenous proteases such that the regions containing the interchain disulphide linkages were lost. This finding reconciles several apparently conflicting reports concerning the occurrence of interchain disulphide bonds in procollagen.

The possibility of a relationship between the process of disulphide bond formation among pro- α chains and that of triple-helix formation was suggested by studies reported by Grant *et al.* (*J. biol. Chem.*, **248**, 7432; 1973) on the biosynthesis of a basement membrane collagen of embryonic chick lens. Procollagen secreted by lens cells was found to be linked by interchain disulphide bonds and in a triple helical conformation. By contrast, intracellular lens collagen was found to be in a random coil conformation, and interchain disulphide bonds between the pro- α chains were also absent. That the formation of the disulphide bonds may be of considerable importance in promoting the formation of the triple helix is also indicated by studies using pulse label and chase experiments in embryonic chick tendon cells (Schofield *et al.*, *Biochemistry*, **13**, 1801; 1974) and cartilage cells (Uitto *et al.*, *Fed. Proc.*, **33**, 617; 1974). A close correlation was observed between the extent of interchain disulphide bonding among pro- α chains and the extent to which the procollagen was in a triple-helical conformation. The rates of these processes, however, seem to vary according to the cell type and may thus influence the time taken for secretion of procollagen from different types of cells.

Further evidence of a role for disulphide bonds in procollagen synthesis comes from the work of Uitto and Prockop (*Biochem. biophys. Res. Commun.*, **55**, 904; 1973). Embryonic chick tendon cells were incubated at 37° C under conditions in which they synthesised and accumulated procollagen, (unhydroxylated procollagen) which is non-helical above 24° C. When the cells were cooled to 15° C, the intracellular procollagen, which was shown to contain interchain disulphide bonds, became triple helical within five minutes. But when disulphide bonds in the amino-terminal extensions of procollagen were reduced by treating the cells with dithiothreitol, the rate of helix formation on cooling was markedly decreased; this suggests that the formation of interchain disulphide bonds is probably a necessary prelude to helix formation. Whether or not this process is mediated by enzymes is unknown, but in tendon cells interchain disulphide bonding of procollagen polypeptides occurs in the rough endoplasmic reticulum soon after release of the completed polypeptides from membrane-bound ribosomes (Harwood *et al.*, *Biochem. biophys. Res. Commun.*, **55**, 1188, 1973).

A further factor in considering the formation of the collagen triple helix is the part played by hydroxyproline. Uitto and Prockop using procollagen were able to dissociate the disulphide bonding process from any influences exerted by hydroxyproline residues in the polypeptides. It is clear from studies of the denaturation temperatures of procollagen and procollagen, however, that the absence of hydroxylated prolyl residues markedly decreases the thermal stability of the collagen triple helix (Jimenez *et al.*, *Biochem. biophys. Res. Commun.*, **52**, 106; 1973; Berg and Prockop, *ibid.*, 115). It seems therefore that the major role of hydroxyproline is to maintain the procollagen polypeptides in a triple-helical conformation under physiological conditions, and that one of the chief roles of the amino-terminal extensions is to facilitate chain association through the formation of interchain disulphide linkages leading to the formation of the triple-helical structure in a time which is biologically feasible.

D. S. JACKSON
M. E. GRANT

First official geological survey in the British Isles

from Gordon L. Davies

IN March 1832 Henry Thomas De La Beche approached the Master General and Board of Ordnance offering to plot geological lines upon the ordnance maps of south-western England, and the outcome was the establishment in 1835 of the Geological Survey of Great Britain as a branch of the Ordnance Survey. A fact which is much less well known is that as early as 1824 Lieutenant-Colonel Thomas Frederick Colby (1784–1852), the Superintendent of the Ordnance Survey, had himself inaugurated an Ordnance Geological Survey of Ireland, and although it proved abortive, it was a pioneering venture that deserves to be remembered in this the year of its hundred and fiftieth anniversary.

Colby was keenly interested in geology (he had been a member of the Geological Society of London since 1814) and he resolved to bring Irish geology within his purview when in 1824 he was directed to extend the Ordnance Survey's operations into Ireland. The Irish rocks were to receive this particular attention because the survey was under instruction to publish maps of Ireland on the lavish scale of 6 inches to the mile instead of the 1-inch scale that was standard in Britain. Colby hoped that as his men worked their way across Ireland they would also be able to collect geological information as a byproduct of their normal topographical duties. Indeed, writing in February 1826 he looked forward to the completion in Ireland of "the most minute and accurate Geological Survey ever published".

Initially Colby's plan was that the surveyors would collect geological samples, mark the collection sites on maps, and then despatch both samples and maps to the survey's Dublin head-

quarters where a geologist would identify the specimens and then interpolate geological lines on the maps. This seems to have been the procedure used when the survey began operations in counties Antrim and Londonderry in 1825, but the inadequacy of such random methods soon became apparent. In November 1826, therefore, Captain John Watson Pringle (who lived about 1793–1861), a Waterloo veteran and a former pupil of Friedrich Mohs at the Freiberg School of Mines, was appointed to reshape the survey's geological activities. Under his direction the field parties were themselves instructed in the construction of geological maps and sections, and for their guidance he prepared a pamphlet entitled *Geological and Mineralogical Observations* fifty copies of which were lithographed at the Tower of London in March 1827. From the surveyors Pringle expected a geological map of each parish accompanied by two geological sections drawn from north to south and east to west, and he estimated that using his methods the geological survey of Ireland would be completed by 1834 at the trifling cost of £5,500.

But Pringle's methods were never accorded a fair trial because Colby's superiors decided that geology was interfering with the survey's more legitimate topographical duties and in September 1828 Colby was directed to cease all geological investigations forthwith. He did manage to revive the Irish Survey's geological activities in January 1830 when Captain Joseph Ellison Portlock (1794–1864) took over the duties formerly discharged by Pringle, and the fruit of this resurrected geological survey was Portlock's classical map and memoir of the geology of Co. Londonderry and adjacent areas published in 1843. By then, however, the Geological Survey of Ireland was merely a poor cousin of the vigorous body that De La Beche had created in England, but this should not be allowed to obscure the significance of Colby's achievement back in the 1820s. He had in 1824 inaugu-

rated the earliest official geological survey to operate within the United Kingdom and that only 3 years after the Corps Royal des Mines had planned in France the earliest of all national geological surveys. The French surveyors, however, did not start work in the field until 1825, the very same year as Colby's men 'broke ground' in Northern Ireland.

Sadly, Colby's abortive geological survey of 1824 never published any of its work, but a few geological maps and sections produced under Pringle's direction have recently come to light in the Dublin offices of the Irish Ordnance and Geological surveys. Some of these items have found a place in the exhibition being staged in Trinity College Dublin to mark the anniversary of the foundation of the Ordnance Survey of Ireland. The exhibition opens on June 7 and will run for 2 months.

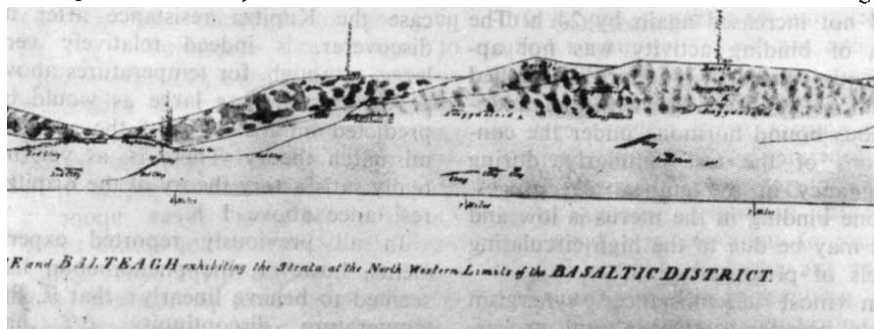
Infrared superhet for detecting pollutants

from our Chemical Physics Correspondent

ANYBODY who, since the 1930s, has built his own radio receiver is aware of the advantages of superheterodyne detection. Not merely can the main amplifier be chosen for a convenient frequency, but more importantly the signal strength is proportional to the product of the electric field strengths of the weak incoming signal and the powerful local oscillator in contrast to the less favourable square of the signal field for direct detection.

Hitherto these advantages have been denied to those who need to detect weak infrared signals, because of the lack of suitable local oscillators whose stability must be good compared with the band width of the amplifier. Stable infrared lasers have altered this position so that the whole problem of the detection of weak infrared emission is being considered. These comments are prompted by an article by Menzies and Shumate (*Science*, **184**, 570; 1974) who use the superhet principle for the detection of small amounts of pollutant gases. But as these authors point out, a wider range of applications is possible including the gathering of meteorological information by sensing the atmospheric emission at selected frequencies. And it may not be long before such detection systems are used in conjunction with astronomical telescopes. Furthermore, when tunable infrared lasers are more readily available the scheme can be used in conjunction with frequency scanning.

The heart of the instrument is the mixer detector, in the present case a copper-doped germanium photoconductor. This receives the signal,



Part of a geological panorama running north to south for 11 miles across the Co. Londonderry parishes of Drumachose and Balteagh. The original possesses a horizontal scale of 6 inches to 1 mile and a vertical scale of about 1 inch to 430 feet. The panorama which shows the Tertiary plateau basalts (blotched) resting upon Cretaceous chalk and Triassic sandstone, is the work of two of Pringle's officers, Lieutenants Lancey and Fenwick, and it is dated November 28, 1827. The topographical detail and geological inscriptions have been lithographed and geological colours then added by hand, and the panorama was evidently used as a specimen designed to show survey parties the type of work expected of them.

chopped with what is effectively a cold chopper, and it also receives 40 mW of power from the c.w. laser at the frequency of interest. The detector is followed by a high radiofrequency amplifier broad banded from 10 to 600 MHz, which amplifies the beat signal between the infrared sources. This device is then sensitive to radiation ± 600 MHz (± 0.02 cm⁻¹) from the laser with the exception of a narrow central gap. Providing in the absence of the emitter gas the source seems to be appropriately cold, a condition ensured in the laboratory by a cold surface beyond the back window of the sample cell, appropriate reference levels may be set and calibration provided.

The gas under study must, of course, emit at the frequency of the laser. Using a CO₂ laser at 1,109 cm⁻¹ (9.02 μ m) for SO₂ samples at room temperature, the emission was as little as 10⁻² atm cm for the product of the partial pressure of SO₂ and the cell length. For ethylene at 950 cm⁻¹ (10.53 μ m), the figure was 5×10^{-3} atm cm in the presence of an atmosphere of nitrogen in each case. There is little doubt that these figures could be appreciably improved with tunable lasers to ensure operation at the peak of the gas line and also if the gas to be detected formed a major component of the sample so that lower pressures could be used.

Oestrogen and progesterone binding

from our
Steroid Biochemistry Correspondent

It is well established that the steroid hormones oestrogen and androgen bind to specific receptor proteins in mammalian reproductive tissues. Binding of progesterone and receptors for it, however, have been more difficult to demonstrate. In rat uterus the progesterone binding protein seems to be the same as the corticosteroid-binding globulin of rat plasma. In guinea pig uterus, however, a progesterone-binding protein (not corticosterone-binding globulin which has only a low affinity for progesterone in this species) is easier to demonstrate.

Two types of binding for oestrogens and androgens exist in the mammalian reproductive tract; one has a high affinity and a limited number of binding sites and, conversely, the other has a low affinity and an unlimited number of binding sites. Progesterone binding in the guinea pig uterus seems to follow the same pattern.

The presence of progesterone receptors has been revealed mainly by

sucrose gradient centrifugation. As in the case of uterine oestrogen receptors, two components seem to exist; one has a sedimentation coefficient of 6.7S and the other has one of 4-5S. In addition to sucrose gradient centrifugation which measures directly the concentration of binding protein, binding of progesterone can be shown by simpler methods involving *in vitro* measurements of bound progesterone after removal of free hormone with adsorbents (Freifield *et al.*, *Steroids*, **23**, 93-103; 1974; Atger *et al.*, *Endocrinology*, **94**, 161-167; 1974).

In both rats and guinea pigs these authors found that treatment with oestrogens increased the uterine binding of progesterone. In the guinea pig the increase began within 4 to 12 h and after 4 days the concentration of high-affinity binding sites per cell showed a ten-fold increase; no further increase occurred if treatment was continued for up to 2 weeks. On stopping oestrogen treatment, binding activity declined with a half life of about 3 days which suggests that the progesterone receptor is a relatively stable protein but that it is dependent on oestrogen stimulation. Further evidence for oestrogenic control of the receptor is the change in the binding protein during the oestrous cycle; the concentration of binding sites is highest at pro-oestrus when oestrogen production is high and the receptor is present mainly in the 6.7S form and lowest at dioestrus when more of the 4-5S receptor is present.

The vagina and uterine cervix of guinea pigs also contains a receptor protein which can bind progesterone. The properties of the receptor in these tissues are similar to those of the uterus although the vaginal receptor seems to be much more dependent on oestrogen than the uterine one.

This effect of oestrogen seems to be antagonised by progesterone. Administration of progesterone to oestrogen-treated castrated guinea pigs caused a rapid decline in uterine binding which reached a minimum by 6 h and which had not increased again by 24 h. The loss of binding activity was not apparently caused by a failure of labelled hormone to exchange with the endogenous bound hormone under the conditions of the test. Similarly, during pregnancy in the guinea pig, progesterone binding in the uterus is low and this may be due to the high circulating levels of progesterone.

In most circumstances synergism exists between oestrogen and progesterone and progesterone only produces a biological response in tissues which have been exposed to oestrogen. This is explainable if oestrogen stimulation is required for the synthesis of progesterone binding protein. The decreased binding activity on pro-

gesterone administration, even when oestrogen is present, might suggest that the action of progesterone is self-limiting—once a sufficiently high level of progesterone in plasma is attained, binding of the hormone in the uterus is decreased. More work is, however, required to determine these relationships.

Anomalous Kapitza resistance

from Peter V. E. McClintock
Condensed Matter Correspondent

THE Kapitza resistance between liquid He II and a solid exhibits anomalous behaviour near 2 K according to a report in *Physical Review Letters* (**32**, 658; 1974) by Cheeke, Hebril, Richard and Turkington of the Centre National de la Recherche Scientifique (CNRS) at Grenoble.

When heat is passing from one solid to another one made of a different material there is always a jump in temperature across the interface which separates them. The phenomenon can be accounted for reasonably well in terms of an acoustic mismatch theory: by analogy with the partial reflection of light which occurs at an interface between two materials with different refractive indices, there is a partial reflection of the lattice waves (phonons), which constitute the flux of heat, as they try to pass between two materials in which the velocity of sound is different. The temperature discontinuity caused by this reflection of phonons is proportional to the heat flux, the constant of proportionality being known as the thermal, boundary resistance.

The velocity of sound in liquid helium is a factor of one twentieth smaller than in most solids, so that the thermal boundary resistance, between liquid helium and a solid would be expected to be particularly large. In fact, the boundary resistance, called in this case the Kapitza resistance after its discoverer, is indeed relatively very large, although, for temperatures above 1 K not nearly as large as would be predicted on the basis of the acoustic mismatch theory. There is, as yet, no really satisfactory theory of the Kapitza resistance above 1 K.

In all previously reported experiments, however, the phenomenon has seemed to behave linearly: that is, the temperature discontinuity ΔT has always been directly proportional to the heat flux Q , within experimental error. The CNRS group has measured, to a high precision, ΔT between copper or lead and liquid helium. They find that, although a plot of ΔT against Q yields an accurate straight line at small values

of Q there is, for any given temperature, a definite heat flux at which the line changes slope by up to 25%. The effect is at its largest near 2 K, gradually diminishing as the temperature is reduced.

It is now more than thirty years since the existence of the thermal boundary resistance was discovered by Kapitza (*J. Phys. U.S.S.R.*, **4**, 181; 1941) and it seems at first sight very surprising that in the course of the large body of experimental work on the phenomenon since that time, no one has reported observation of the anomalous features now being described. The effect would clearly be easily missed, however, except in cases where a large number of closely spaced data points were taken at small values of Q . Ironically, there is perhaps some evidence for the existence of these anomalies in the original graphs published by Kapitza.

As for the origin of the anomalies, the authors discuss a number of possibilities the most plausible of which concerns the onset of turbulence in the helium very close to the metal surface. Liquid helium at these temperatures, He II, can be regarded as an intimate mixture of two interpenetrating but extremely dissimilar fluids: a normal fluid component which behaves much like any other liquid and which carries all the thermal energy of the helium and a superfluid component which carries no thermal energy, has zero viscosity and is thus responsible for many of the more bizarre properties of the liquid. When heat enters the liquid, a counterflow of the two components occurs, with superfluid approaching the heated metal, being converted into normal fluid and then flowing away again. The authors tentatively conclude that the observed anomalous behaviour arises from the onset of turbulence in the very thin layer of helium at the surface of the metal, but further work will be necessary before this interpretation can be confirmed.

The authors have taken considerable pains to try and ensure that they have not been observing a mere artefact arising from their measuring equipment or experimental techniques. If the phenomena which they report are genuine, however, any theory of Kapitza resistance under these conditions will need to take them properly into account, so it seems highly desirable that the experiment be repeated in another laboratory as soon as possible.

Protein folding made simple

from Barry Robson

It is hardly surprising that nobody has yet predicted how a given protein

molecule folds up, considering that there are very much simpler molecules whose conformations defy theoretical analysis. Theoreticians with insatiable appetites for protein molecules, however, can restore at least a modicum of common sense by first examining protein systems in which prediction of the folding process is probably a simpler task.

Ptitsyn and Rashin (*Dokl. Acad. Nauk SSSR*, **213**, 473; 1974 and *Bio-phys. Chem.*, in the press) have made the first attempt to predict the folding and final overall conformation of a globular protein from its amino acid sequence and known secondary (locally organised) structure. The relatively simple protein system is in this case the myoglobin molecule which along with its haemoglobin relatives, is exceptional in being composed of α helix and very little else. Ptitsyn and Rashin justify starting with the known helical regions in terms of the popular model in which such regions are among the first features to appear during the folding up of the polypeptide chain, a model which is consistent with experimental evidence (Jardetzky *et al.*, *Cold Spring Harb. Symp. quant. Biol.*, **36**, 257; 1971; Robson and Pain, *Proc. Jerusalem Symp. quant. Chem. Biochem.*, **5**, 279; 1973; Sachs *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **69**, 3790; 1972). Furthermore, recent publications confirm that α -helical regions in proteins can be predicted with reasonable accuracy (see, for example, Chou and Fasman, *Biochemistry*, **13**, 222; 1974).

The method of Ptitsyn and Rashin is to try and predict the folding pathway and resulting most stable configuration of stacked α helices on the basis of hydrophobic interactions between the helices. They find that the most stable configuration "coincides in a rough resolution" with the native conformation. It is surprising, however, that they obtain even such an approximate structure, and it is necessary to ask whether such an agreement could be fortuitous. Helices were treated simply as cylinders with a radius of 5 Å, and dispersion, electrostatic and hydrogen bonding forces between helices are neglected except to the extent that they are included in the parameters of the hydrophobic interactions. Furthermore, a possible contribution of the helix backbone to the hydrophobic interactions was not investigated. Finally, the study was apparently carried out manually and because of the still very large number of possible configurations it would be difficult to exclude an accidental subjective element from entering such calculations where the answer is known in advance.

It is therefore possible that in retrospect the simplifications of Ptitsyn and Rashin will be seen to be too simple,

and their work is likely to be best remembered for the more critical reassessments of the problem which it will undoubtedly stimulate. Nevertheless, the concept of interactions between secondary structure features such as α helices leading to the formation of single compact globules is a potent one and extends well beyond the simple case of myoglobin. The recent work of Rossman and Liljas (*J. molec. Biol.*, **85**, 177; 1974) confirms and quantifies the idea that sections of the backbone of more complicated proteins are bunched up locally to form subglobules within the overall globular structure of the molecules, so dividing many protein molecules into two or more distinct lobes. Within such subglobules, interactions between α -helical regions and other secondary structure features are probably very important.

As the first protein to have its conformation determined by X-ray crystallography, myoglobin once surprised biochemists with its unexpected complexity and the lack of any obvious relation between amino acid sequence and conformation. Now the pendulum is beginning to swing from a state of general pessimism to a state of optimism which is probably not unjustifiable.

All excited in Lisbon

from a Correspondent

THE opulent ambience of the Calouste Gulbenkian Foundation Centre in Lisbon on April 18–24 was the choice for an international conference on excited states of biological molecules under the sponsorship of the European Physical Society and European Photochemical Association, with J. B. Birks (University of Manchester) as papers secretary and editor.

As both Sir George Porter (Royal Institution, London) and G. Eisinger (Bell Laboratories, New Jersey) noted in their lectures, 1974 is about 25 years since the seminal Faraday Society Discussion of 1950, when M. Kasha discussed the phosphorescence of organic molecules in terms of the Jablonski diagram and singlet-triplet conversion, and about 50 years since Perrin postulated singlet-singlet energy transfer to explain the depolarisation of fluorescence. Excited states in biological molecules were last discussed at an international meeting at Arden House, New York, in October 1969 (see *Nature*, **226**, 113–118; 1970). In the light of that report, the programme of this year's meeting had inevitably a slight sense of *déjà vu*, as the main themes have not changed greatly in 5 years.

Porter reviewed the methods avail-

able for the direct observation of excited states, with particular reference to nanosecond and picosecond laser flash photolysis. He emphasised the importance of substituent effects on the reactivity of radical species formed from excited-state ketones, and the biological roles of plastoquinone and ubiquinone, which are the subject of current work in his laboratory.

C. Hélène (Centre National de la Recherche Scientifique, Orléans) gave a lucid review of excited state interactions and energy transfer processes in oligopeptide-nucleotide complexes. He described energy transfer between stacked nucleotides and aromatic amino acid chromophores in frozen aggregates at both singlet and triplet levels. It is necessary to intercalate the bound aromatic residue between the bases of single-stranded nucleic acid for quenching of fluorescence. The ability of small peptides containing an aromatic residue to bind to a small, single-stranded region of DNA containing, for example, a thymidine dimer, followed by photosensitised splitting of the dimer by energy transfer from the excited aromatic residue, provides an elegant example of a specific photoprocess in a model protein-DNA complex.

In a lecture entitled "What We have learnt about Proteins from a Study of their Excited States", G. Weber (University of Illinois) cited the demonstration of processes in the nanosecond or shorter time range, such as fast degradation of excess vibrational energy of the excited state, energy transfer between aromatic chromophores, solvent relaxation effects, molecular or segmental relaxation times and the correlation between free residue rotation and protein unfolding. He considered that with the advent of time-resolved spectroscopy on a nanosecond time scale, future work would be largely concerned with the dynamics of excited state processes. The use of differential phase fluorimetry to study solvent relaxation was described, and also the use of dissolved oxygen (under high pressure in a special cell) as an uncharged fluorescence-quenching agent. From comparisons between plots of fractional fluorescence and fractional lifetimes it is possible to determine if the quenching is homogeneously dynamic, or static and involving complexes. Of the proteins examined, some show deviations from pure dynamic quenching.

The lecture by H. T. Witt (Max-Volmer Institute, Berlin) was an impressive example of the value of a conceptual model in planning definitive experiments in such a complex field as photosynthesis. Witt demonstrated how the specific structural arrangements of the functional membrane (thylakoid) of the photosynthetic sys-

tem mediates extraordinary reaction patterns not seen *in vitro*. He presented evidence in support of the following sequence of primary acts of energy conservation and utilisation: (1) the channelling of light absorbed by bulk pigment (carotenoids and chlorophylls) by singlet-singlet transfer to photoactive chlorophyll, with a triplet-triplet transfer path from chlorophyll back to carotene to protect the former from irreversible photo-oxidation at high light intensity; (2) a specific arrangement in which always two photoactive chlorophylls are coupled in series in the membrane and are able to pump electrons from H_2O to $NADP^+$ to form NADPH; (3) the creation of a charge across the membrane; (4) the discharge of the membrane potential by H^+ fluxes through specifically organised channels (ATPase) coupled with the generation of ATP from $ADP+P$. It is the NADPH and ATP formed in the system which carry out the reduction of CO_2 into sugar and "everything else". The membrane potential is sufficiently high to give rise to a Stark shift in the absorption spectra of adjacent chromophores, and the shift can be regarded as a "molecular voltmeter". Some ingenious experiments to demonstrate this engaging concept were described. It seems that at least four key features of Witt's model of vectorially directed processes in the photosynthetic membrane have been confirmed experimentally.

Eisinger described a more critical approach to the interpretation of singlet energy transfer by the Förster mechanism. Since the transfer efficiency between two luminophoric groups on a macromolecule depends not only on the distance (R) between them, but also on the relative orientation of their transition dipoles, the value used for the orientation factor (κ^2) is crucial for the calculation of R . The widely-used value of $2/3$ can be derived for the dynamic random averaging case, which, however, is unlikely to be relevant to real macromolecule situations. Between the extremes of fixed relative orientation, κ^2 varies from zero to 4. Eisinger and Dale have calculated contour maps showing the variation of κ^2 as the transition dipoles are given orientational freedom within cones of specified half-angles for a variety of donor-acceptor group geometries. Their analysis shows it is fallacious to assume that in practice κ^2 values near the extreme values are unlikely. They estimate the extent of orientational freedom from the limiting emission anisotropies of the separate luminophores (from time-dependent polarised emission data), and hence get reasonable upper and lower bounds for κ^2 . Since information on the relative orientation of the donor-acceptor pair can be ob-

tained from the depolarisation of transferred energy, a realistic estimate of κ^2 is possible. This procedure was used to obtain an R value for energy transfer from the fluorescent Y-base of the anticodon loop of a t-RNA to a bound dye at the remote end of the macromolecule.

S. A. Latt (Harvard Medical School) used fluorescent probes to study chromosome structure. The fluorescence of bound quinacrin is quenched by guanine-cytosine base pairs of the DNA, so that the fluorescence reveals regions with high adenine-thymine content. Furthermore, the base analogue 5-bromodeoxyuridine biosynthetically incorporated into DNA quenches the fluorescence of the dye Hoechst 33258, and can therefore be used to study the time course of DNA replication in chromosomes, and also some aspects of chromosome structure and stability.

L. G. Christophorou (Oak Ridge National Laboratory and University of Tennessee) described how threshold electron excitation spectroscopy, based on the resonant interaction of slow electrons with vapours, can be used to detect optically-forbidden transitions in aromatic compounds, including triplet states and transient negative ions. The stabilities of the double negative ion resonant states can be simply correlated with established substitution parameters of the benzene ring.

In an interesting application of luminescence spectroscopy, J. W. Longworth (Oak Ridge National Laboratory) reported fluorescence and phosphorescence data for more than 50 Bence-Jones proteins — 'light chain' dimers of γ -immunoglobulins which are secreted as individual variants in myeloma. The light chain is believed to fold in a homologous conformation, the immunoglobulin fold, and comprises two domains of approximately equal size, the variable N-terminal domain, and the C-terminal domain which is constant in all light chains of a given type. The observed diversity of fluorescence, with respect to amplitude, spectral distribution and lifetime, can be ascribed to perturbation of a single tryptophan residue in the variable domain. The phosphorescence spectra are equally diverse, often showing two decay components. Bence-Jones proteins therefore provide an excellent opportunity for studying the effects of limited variations in residue composition on the luminescence properties of a specific protein structure. There is some evidence that interaction between tryptophan and a contiguous disulphide bond is responsible for the short-lived phosphorescence, and some other contributors also considered the possible role of such an interaction in protein luminescence spectroscopy.

I. Tatischeff (Institut du Radium-

Laboratoire Curie, Paris) reported variations of the fluorescence quantum yield of the three aromatic amino acids with excitation wavelength. For all three, the yields, based on both quinine sulphate and sodium salicylate as wavelength-independent quantum counters, were constant in the longwave absorption bands, then fell abruptly to lower constant values in the shortwave absorption bands. The discussion provoked by her findings was about equally divided between explaining it away as due to experimental artefacts or inadequacy of the quantum yield standards, and in accepting them as correct and proposing possible explanations.

From the rather small number of communications concerned with small peptides and polypeptides, that by Ph. Wahl (Centre de Biophysique Moléculaire, Orléans) on the pulse fluorometry of tryptophyldiketopiperazine may be noted. He concluded that quenching of tryptophan fluorescence by the peptide bond only occurs in a folded conformer. The equilibrium constant relating the folded and unfolded conformations seems to be different in the excited and ground states; the ground state value is available from nuclear magnetic resonance studies.

An indication of new spectroscopic techniques was the inclusion of contributions on magnetic circular fluorescence and fluorescence polarisation and on magnetic circular dichroism in the near infrared. The shorter papers on visual pigments were concerned with problems of singlet-triplet intersystem transfer and *cis-trans* isomerisation of polyenes, and in the large number of communications on photochemical reactors, the roles of singlet and triplet oxygen were frequently discussed.

Management of aquatic weeds

from Peter D. Moore
Plant Ecology Correspondent

At the close of the International Hydrological Decade it is only natural that the world of waterweeds and the problems associated with their control should be receiving some attention in the scientific literature. It is natural too that the problems of the developing countries should receive greatest prominence, for here many essential programmes of irrigation are being set back by the luxuriant and rapid growth of aquatic vegetation in tropical climates. India, most especially, has been at the fore in a series of recent publications on this subject.

Gupta has summarised many of the Indian problems and some of their solutions in his booklet *Aquatic Weed Control* (Rajasthan College of Agri-



Weed Research Organisation

Water channel in India infested with the water hyacinth *Eichornia*.

culture, Udaipur, 1973). In Rajasthan the development of a canal system is relieving a serious problem of water distribution; many women were forced to walk several kilometres to fetch pitchers of water from surface ponds. The canal projects, however, have suffered severely because of the growth of aquatic weeds, particularly water hyacinth, *Eichornia*, and the water ferns *Azola*, *Wolfia* and *Salvinia*. From April to June the canals are closed so that clearance by hand can take place, but this control method is ineffective in the long term. Only one mechanical weed cutter operates in Rajasthan but biological control using Chinese carp is being attempted; the use of chemical herbicides is prohibitively expensive. The same sad picture of India's plight emerges from the recent UNESCO symposium held in New Delhi and reported by Tinker (*New Scientist*, 61, 747; 1974).

A very full review of the entire problem has now been published by UNESCO (*Aquatic Vegetation and its Use and Control*, edit. by D. S. Mitchell, Paris, 1974). Although the theoretical consideration of the mathematics of waterweed population growth and the technique of mechanical, chemical and biological control of waterweeds are undoubtedly important as short term approaches to the aquatic vegetation problem, data presented by Boyd (University of Auburn, Alabama) in this collection of papers provide hope for a more permanent solution. The explosive growth of aquatics is itself an indication of eutrophication and nutrient wastage which can ill be afforded, especially in countries such as India. Boyd considers ways in which waterweeds could assist in the retrieval of these last elements.

Many waterweeds provide a better feed for livestock than dried alfalfa hay. Generally they contain rather less

crude protein and fibre, but more ash and fat than this commodity. Inorganic ion concentration in aquatics falls within the range generally accepted for crop plants; ash values of more than 25% of the dry weight are recorded, the noxious weed of many Indian waterways, *Hydrilla verticillata*, being responsible for some of the higher figures.

Before these nutrients can be re-used by crops they must be released from the organic tissues of the water plants, and decomposition is not always rapid. *Eichornia* compost takes about 3 months of fermentation before it is suitable for use as a fertiliser. Nevertheless, when it is considered that eutrophicated waters can support a standing crop of 29 t ha⁻¹ (dry weight) of *Eichornia* containing 157 kg ha⁻¹ of phosphorous and 693 kg ha⁻¹ of nitrogen, this process must be considered an important means of cutting nutrient losses.

In the developed countries the cost of retrieving lost nutrients from solution usually exceeds the cost of supplying new ones. In countries such as India, where manual harvesting is cheap and where the foreign exchange needed for chemical fertilisers is in short supply, the use of aquatic vegetation for nutrient recovery may prove to be economically feasible.



Weed Research Organisation

Pistia stratiotes.

What is the nuclear optical potential?

from Peter E. Hodgson, Nuclear Physics Laboratory, Oxford

THE interaction of a nucleon of moderate energy with a nucleus is an exceedingly complicated process. Very many reactions can occur and they depend on the energy levels of the compound and residual nuclei. The conservation of particle flux links all the reaction channels together, so that any abnormal behaviour in one channel, a resonance or threshold for example, produces corresponding changes in all the others. It might well be thought that each interaction would have to be treated as a special case and that the detailed structure of each nucleus would have to be taken into account before the cross sections could be calculated.

One-body potential

In spite of this somewhat pessimistic expectation, the striking regularities in the neutron total and reaction cross sections as a function of energy and from nucleus to nucleus suggested that they could be described by a one-body potential varying smoothly with energy and nuclear size. (Feshbach, Porter and Weisskopf, *Phys. Rev.*, **96**, 448; 1954). The success of this description led to the model being extended to nucleon elastic scattering and the range of other nuclear interactions.

Subsequently it was realised that this success was partly due to the relatively poor energy resolution of the early measurements. Later improvements in experimental techniques showed that the cross sections of reactions passing through the compound nucleus do fluctuate as the incident energy varies (Von Brentano *et al.*, *Phys. Lett.*, **9**, 48; 1964) but if the energy spread of the incident beam is greater than the mean width of the fluctuations an averaged cross section is obtained that generally varies quite smoothly with energy. Detailed analysis showed that this averaged cross section is the sum of compound nucleus and direct reaction components; the former may be calculated using the theory of Hauser and Feshbach (*Phys. Rev.*, **87**, 366; 1952) and subtracted from the measured cross sections. As the energy increases the contribution of the compound nucleus processes falls rapidly and soon becomes negligible.

In precision analyses it is necessary to remove the compound nucleus contribution to the data by a preliminary

Hauser-Feshbach calculation or preferably to confine the analysis to energies for which the compound nucleus contribution is negligible. For most nuclei, energies of about 15 MeV or more are adequate for this purpose. The direct cross section is then analysed by a simple optical potential.

Many such analyses were made and soon it was possible to specify potentials defined by rather few parameters that give strikingly good overall fits to a wide range of neutron and proton elastic scattering and reaction data (Perey, *Phys. Rev.*, **131**, 745; 1963; Perey and Buck, *Nucl. Phys.*, **32**, 353; 1962). One of the most successful of these potentials was obtained by Perey from an analysis of the elastic scattering of protons of 9–22 MeV by a range of medium and heavy nuclei. He used a potential of the form

$$V(r) = U_0 f(r, a_u) + i a_D W_D(d/dr)[f(r, a_D)] + (\hbar/m\pi c)^2 U_s(l/r)(d/dr)[f(r, a_s)] L \cdot \sigma$$

where

$$f(r, a_i) = [1 + \exp\{(r - r_i A^{1/3})/a_i\}]^{-1}$$

In the first analyses, the parameters of this potential were adjusted to optimise the fit to each set of data for scattering from a particular nucleus at a particular energy. The most accurate data were the differential cross sections, supplemented by less accurate polarisations and reaction cross sections.

The optimum parameters for different nuclei at different energies were found to be very similar, so the data were reanalysed fixing the form factor parameters to the values

$$r_u = 1.25; \quad r_D = 1.25; \quad r_s = 1.25 \\ a_u = 0.65; \quad a_D = 0.47; \quad a_s = 0.65.$$

The quality of the fits was still very high, though naturally not quite as good as previously.

The depth of the real potential was found to vary systematically with energy and with the numbers of neutrons and protons in the nucleus, and the variation could be well represented by

$$U = 53.3 - 0.55E + 27\alpha + 0.4Z/A^{1/3}$$

where $\alpha = (N - Z)/A$

The optimum imaginary potential was not so well determined, but a good average value is

$$W_D = 12A^{1/3}$$

The polarisations were best fitted with

$$U_s = 7.5 \quad (E < 17); \quad U_s = 8.5 \quad (E > 17)$$

The fits obtained with this overall potential are very little worse than those obtained when several parameters were adjusted in each case.

Global potential

The striking success of these analyses led to the hope that it would be possible to find an overall or global potential for all nuclei and one has tended to forget that the individual structure of each nucleus must affect the interaction to some extent. This raises difficulties concerning the concept of the optical potential, in particular whether it should give a good overall fit to many sets of data or whether it should be adjusted to fit each set of data as accurately as possible.

Optical potentials of the first type are the same for all nuclei, with perhaps a smooth and easily parameterised dependence on the incident energy and on Z and A . To achieve this one has inevitably to make some sacrifice in the quality of the fits to the data.

Optical potentials of the second type have parameters that are unique to each nucleus at each energy, so that accuracy has been purchased at the expense of loss of generality.

Both types of potentials have their advantages and disadvantages. In some interactions there are quite substantial deviations from the behaviour given by the overall potential, whereas potentials fitted accurately to particular sets of data are sometimes recognisably non-physical. These anomalies have a physical basis, so that one might hope that detailed studies with both types of potential could teach us more about the physical process taking place.

It is important to see what can be done to eliminate the disadvantages of these potentials while preserving their good points. The first step is to identify the physical processes responsible for the deviation from 'normality' and make a physical model that allows them to be calculated explicitly. If this can be done within the framework of the optical model, then the process and its effect on the cross section can be represented by an extra term in the optical potential. In this way, for example, the polarisations of the elastically scattered nucleons were included in the model by the addition of the spin-orbit term to the potential and the dependence on the asymmetry parameter by the term proportional to $(N - Z)/A$.

Small terms

At one stage it was hoped that all new physical effects and deviations from the simpler potential could be brought within the general optical potential by the addition of a series of small terms, giving a potential expressible as a simple analytical function of A and E and capable of fitting the elastic scattering data to high accuracy.

Unfortunately in recent years several deviations from normality have been found that can be understood physically but which cannot be treated in this way. These are of two types; the first can still be treated within the optical model formalism whereas the second requires more sophisticated theoretical treatment.

An example of the first is the exceptionally low neutron reaction cross sections found for magic nuclei at low energies, especially for ^{208}Pb (Perey and Buck, 1962). These are much less than those calculated from the highly successful Perey-Buck potential and can easily be understood as a consequence of the low level density at small excitation energies of such nuclei. The effect can be treated phenomenologically by allowing the absorbing part of the optical potential to depend on the shell structure but the absorbing potential cannot conveniently be expressed as an analytical function of Z and A . This phenomenon only occurs in a limited energy region and is not of general importance.

The effects in the second category may be calculated by appropriate physical models but it is not possible to represent them by an easily-parameterised additional term in the optical potential. It is of course often possible to fit the data through the region of the anomaly with a particular optical potential, so that one can formally define the potential responsible for the effect as the difference between the general optical potential and the particular potential, but this is often highly singular and not conveniently parameterisable.

The extent to which this occurs implies a breakdown of the simple optical model, so that one is now faced with the alternatives of either using an ill-behaved effective potential in the framework of the simple optical model or of continuing to use a general optical potential in a more sophisticated formal framework. The second alternative is clearly the more desirable, even from the purely phenomenological point of view, as it often unifies a range of phenomena in a simple way, whereas the effective potentials are much more difficult to handle.

This may be illustrated by the analysis of elastic scattering from strongly deformed nuclei. These have low lying collective states that are readily excited by inelastic scattering and the cross sections are so large that the elastic scattering cross section is significantly affected.

The inelastic cross sections vary in magnitude with β , but hardly at all in shape, and the elastic cross section departs more and more from its unperturbed value as β increases. If the elastic cross section is analysed for

several deformed nuclei, the parameters of the resulting potential vary from nucleus to nucleus depending on the strength of the coupling to the collective states.

In particular, the imaginary potential is found to increase with increasing deformation. A coupled-channels analysis can, however, fit the elastic cross section with the same optical potential for each nucleus provided each has its characteristic deformation parameter. This was first shown by Perey (in *Nuclear Spectroscopy with Direct Reactions* (edit. by Throw) ANL-6848, 114; 1964) in an analysis of the elastic scattering of protons of 17 MeV off some heavy spherical and deformed nuclei.

Another example is provided by the scattering of α particles of 50 MeV by the even isotopes of samarium (Glendenning, Hendrie and Jarvis, *Phys. Lett.*, **26B**, 131; 1968). The elastic scattering changes qualitatively from one isotope to the next; the diffraction oscillations become less and the envelope of the maxima steeper as the mass (and the nuclear deformation) increases. Simple optical model analyses of these data give quite different potentials, but the cross sections are all well fitted by the same optical potential when the coupling to the collective states is taken into account and each nucleus is characterised by its deformation parameter. The deviations from simple normality can thus be brought within the scope of the general optical model, although the deformation parameter cannot be expressed as an analytical function of Z and A .

Coupled-channel analyses

Further evidence for the reduction of the absorbing potential in the region of closed shells is provided by measurements of the s and p -wave strength functions. These show characteristic maxima and minima as a function of A and the overall behaviour is quite well given by the optical model with standard potentials. Closer examination shows that although the maxima are quite accurately fitted, the experimental values are much less than the calculated ones in the regions of the minima. A coupled-channels analysis by Newstead (in *European Conference on Intermediate Reactions*, Plitvice; 1972) found that this behaviour can be obtained by reducing the imaginary potential in a way that varies from nucleus to nucleus. Since the imaginary potential corresponds to absorption it might be expected to be correlated with the number of three-quasiparticle states that can be formed in the initial stages of the nuclear excitation. Newstead estimated the numbers of such states in various nuclei and found that they correlate very well with the imaginary

potentials W obtained by requiring a fit to the s -wave strength function data. Thus once again the deviations from normality can be explained by a physical model but not in a way that can easily be parameterised in terms of A and Z .

Another effect that can easily be treated by the coupled-channels formalism is the marked resonances that occur when low energy nucleons are scattered by some light nuclei (Mikoshiba, Terasawa and Tanifugi, *Nucl. Phys.*, **A168**, 417; 1971). Some of these are single-particle resonances in the optical potential; others can be understood as core-excitation resonances. The coupled-channels formalism with a simple model of the nuclear excitation gives a good overall account of these resonances. The data also show higher order resonances that could presumably be treated by including the coupling to more complicated configurations.

The scattering from light nuclei at higher energies frequently shows deviations from normality and in some cases this is due to higher order processes. In the case of medium-energy proton scattering by ^{12}C and ^{16}O the core-polarisation exchange mechanism with intermediate excitation of a giant resonance is responsible for the marked peaks in the backward cross section for inelastic scattering to non-normal parity states that cannot be accounted for by the simple optical model (Von Geramb, *Nucl. Phys.*, **A199**, 545; 1973; *Nuovo Cim. Lett.*, **5**, 333, 1972). This effect reflects back on the elastic channel and so should be taken into account explicitly at those energies where giant resonances are important.

As a final example, the (p, n) quasi-elastic reaction to the isobaric analogue state has significant contributions from the two-step (p, d, n) intermediate particle transfer process (Rickertsen and Kunz, *Phys. Lett.*, **47B**, 11; 1973). If it is analysed with a simple optical potential the fits are not strikingly good and the parameters of the isospin term which is responsible for the transition vary erratically from nucleus to nucleus. If the two-step process is included explicitly, making use of the properties of the known intermediate states, then the fits are significantly improved and the isospin potential is essentially the same for all nuclei. Thus once again by treating explicitly the process responsible for the deviations it is possible to recover a general optical potential.

Polarisations

The polarisations of the elastically scattered particles are much more sensitive to the interaction potentials than are the differential cross sections, as they depend on the differences of scattering amplitudes. This sensitivity

is to a large extent offset by the lower accuracy of the polarisation data, but nevertheless careful analyses of polarisations have shown anomalies that are not yet understood.

An example of this is provided by the work of Boschitz (*Phys. Rev. Lett.*, **17**, 97; 1966), who analysed a series of differential cross sections and polarisations. He was able to obtain good overall fits to the polarisation data but the optimum values of the parameters of the spin-orbit term in the optical potential showed marked variations from one nucleus to another and as a function of proton energy. So far there is no convincing explanation of this behaviour.

From these examples it will be seen that the processes that must be considered explicitly are of several types. The polarisation and the dependence on the nuclear asymmetry can easily be incorporated in the optical model. The others require quite sophisticated calculations before the effects due to the general optical potential and the special process can be disentangled. The calculations with both processes included give directly the elastic cross section and there is no effective optical model potential as an intermediate stage. It is thus not possible to think of a general optical potential in which the effects of coupling to excited states, two-step reactions, core-polarisation exchange processes and so on are included by appropriate analytical terms in a general expression of the optical potential. In this sense the optical potential ceases to be a concept able to give a detailed physical account of all the data; it has been superseded in some regions by more sophisticated calculations that give an accurate account of the data that cannot be obtained in any other way.

One has thus arrived at a third concept of the optical potential as one that includes as many effects as possible in the parameterisation of its parameters as functions of Z and A but which has to be used in a more sophisticated formalism if certain other effects are to be treated adequately. Thus some of the simplicity of the model has been lost, but further insight into more complicated phenomena has been attained.

I thank Dr R. S. Mackintosh for discussions and suggestions.

Correction

IN the News and Views article "Vegetational change in an aboriginal environment" (*Nature*, **249**, 11; 1974), the date in line 7 of the first paragraph should be 5,500 radio-carbon years.

Biogenesis of surface membranes

from R. Colin Hughes

ACCORDING to the membrane flow theory, membrane biogenesis involves the physical transfer of membranes from one subcellular compartment to another. The endoplasmic reticulum is the precursor of Golgi membrane which is in turn processed into plasma membrane. Surface membrane proteins and glycoproteins, therefore, are considered as a special class of secreted substances.

The basic assumption of the theory, that membrane proteins are synthesised on the rough endoplasmic reticulum, must obviously be re-interpreted, however, since Lodish (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 1526-1530; 1973) and Lowe and Hallinan (*Biochem. J.*, **136**, 825-828; 1973) have described synthesis of some membrane proteins on free polysomes. The converse proposal that soluble cytoplasmic proteins are made only on free polysomes is similarly an oversimplification. At least one soluble enzyme, serine dehydrase, is synthesised on membrane-bound ribosomes and a similar role seems likely in non-secretory tissues such as brain or muscle. So far as membrane proteins are concerned, it is possible that integral membrane components are made in a way different to extrinsically bound proteins. Synthesis of glyceraldehyde-3-phosphate dehydrogenase on cytoplasmic polysomes is expected, for example, yet this protein forms stable interactions with high affinity for a limited number of sites on the cytoplasmic face of the erythrocyte membrane (Kant and Steck, *J. biol. Chem.*, **248**, 8457-8464; 1973). The enzyme seems therefore to be a specific membrane component, made on free polysomes.

An indication that integral proteins, by contrast, may follow an intracellular route analogous to the secretory process comes from an extensive radioautographic study by Leblond and his colleagues (*J. Cell Biol.*, **60**, 258-284; 1974). This suggests a final step in surface membrane biogenesis in which fusion takes place between the cell surface membrane and Golgi vesicles, the membranes of which carry proteins and glycoproteins destined ultimately for the surface membrane. The chief difficulty in accepting completely this proposal, is that the membrane composition of highly purified rat liver Golgi membranes, as described recently by Palade *et al.* (*J. Cell Biol.*, **59**, 45-72; 73-88; 1973) is clearly different to the homologous plasma membrane. The role of the Golgi vesicles therefore, may be to ferry a few proteins from intracellular smooth membranes to the cell surface as follows: fusion

of the vesicles with the cell surface membrane, lateral diffusion of selected proteins into the area of plasma membrane surrounding the fusion site and finally re-entry of the Golgi vesicular membrane into the cell by endocytosis. In this case, the membranes of Golgi vesicles before fusion would contain a relatively small proportion of plasma membrane components superimposed on a background of purely Golgi membrane proteins and after fusion only Golgi specific proteins.

The membrane flow theory also predicts that at earlier stages of membrane biogenesis, the proteins and glycoproteins are restricted to membrane-bounded intracellular vesicles. Presumably, the membrane precursors made on membrane-bound ribosomes are either discharged into the lumen of the endoplasmic reticulum to be inserted into smooth membranes elsewhere or remain inserted directly into membranes at their sites of biosynthesis. Hirano *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **69**, 2945-2949; 1973) found earlier that the membranes of rough microsomal vesicles are assymmetrically substituted with carbohydrate groups, capable of reacting with certain lectins coupled with ferritin. The membrane facing inwards carried carbohydrate whereas the cytoplasmic face did not. Since the intraluminal face of the endoplasmic reticulum is equivalent to the external face of the plasma membrane, at which carbohydrates are also localised, this finding is interpreted as a flow of membrane with retention of configuration from intracellular biosynthetic sites to the cell surface. It does not, of course, prove that this is the case. Kreibach *et al.* (*Fed. Proc.*, **32**, 2133-2138; 1973; *J. Cell Biol.*, **60**, 616-627; 1974) now confirm these observations by testing the accessibility of rat liver microsomal proteins to lactoperoxidase labelling. The glycoproteins are labelled only when the closed microsomal vesicles are made permeable by treatment with low concentrations of detergents. Two classes of glycoproteins are labelled; one class is released by mild detergent treatment whereas the other requires higher detergent concentrations and disassembly of the microsomal membranes. It is not clear, however, whether these last components are stable membrane constituents or precursors of glycoproteins.

The glycoproteins detected by Hirano *et al.* and Kreibach *et al.* are not known to be precursors of surface membrane glycoproteins and could equally well be glycoproteins destined for secretion. The next step is application of the techniques for the selective solubilisation of microsomal glycoprotein precursors to defining the kinetics of flow of these substances.

Gangliosides as membrane receptors for tetanus toxin, cholera toxin and serotonin

W. E. van Heyningen

Sir William Dunn School of Pathology, University of Oxford, UK

Tetanus toxin is bound by a sialidase-labile disialosyl ganglioside, serotonin by another, and cholera toxin by a sialidase-stable monosialosyl ganglioside. There is good evidence that the monosialosyl ganglioside may be the receptor for cholera toxin in cell membranes; what of the disialosyl ganglioside and tetanus toxin?

THERE has been a sharp rise in interest in the possible biological roles of the gangliosides, discovered by Klenk in 1935–42^{1,2}. These versatile substances have been virtually ignored in recent authoritative reviews on the structure of cell membranes^{3,4}, in spite of a number of characteristics which indicate that they might be considered as possible functional components of cell membranes. First, they have an amphiphilic nature, as they are composed of lipophilic ceramide moieties and hydrophilic oligosaccharide moieties; second, in nervous tissue, which is the richest source of gangliosides, they have found to be concentrated in fractions containing synaptic membranes^{5,6}; third, they are being found in an increasing number of biological preparations⁷; fourth, although they are water soluble they are almost invariably not extractable with water from biological material, which indicates that they are bound to hydrophobic components^{8,9}.

(Bretscher⁴ proposes that the outer layer of the membrane lipid bilayer is largely composed of ceramidyl phospholipids, and that the glycolipids are located solely in this layer. In such a scheme it would be reasonable to speculate that the ceramidyl moieties of the gangliosides may be buried, leaving the hydrophilic oligosaccharide moieties exposed on the outsides of the membranes.)

No particular function for the gangliosides has, up to now, been clearly demonstrated, but recently there has been an accumulation of evidence that cholera toxin may recognise a particular ganglioside on the surfaces of cell membranes.

These observations revive interest in earlier observations of a specific interaction between tetanus toxin and two other gangliosides, and between serotonin and another ganglioside.

Tetanus toxin

Tetanus toxin is a neurotoxin, thought at first to act only on the central nervous system (CNS), producing spastic paralysis by blocking synaptic inhibition in the spinal cord, but now known to act peripherally as well, producing flaccid paralysis by blocking neuromuscular transmission^{10,11}. In its action on nervous tissue, it produces no morphologically distinguishable changes in the tissue, yet it binds with the tissue. This was first shown in 1898 by Wassermann and Takaki who found that when a homogenate of nervous tissue was mixed with a solution of tetanus toxin and the suspension filtered, the concentration of toxin in the filtrate was diminished¹². This phenomenon was investigated by many workers, including Landsteiner, who believed that the substance in nervous tissue responsible for the binding was a cerebroside, but was puzzled by the fact that grey matter had a greater capacity to bind the toxin than white matter, but had a lower cerebroside content¹³. I reinvestigated the Wassermann-Takaki phenomenon and showed that it was apparently tissue specific and toxin specific (that is, of all the tissues tested only nervous tissue bound the toxin, and of all the known bacterial toxins tested only tetanus toxin was bound¹⁴). When these tests were reported in 1959 cholera toxin was not yet known, its existence being reported by De in the same year¹⁵.

It was then shown that the binding of tetanus toxin was due to the then comparatively unknown substance, ganglioside. It was also shown that there were at least three gangliosides in nervous tissue, differing in their sialic acid contents and their

tetanus toxin-binding capacities, which increased with increasing sialic acid content¹⁶. Later, when the multiplicity and nature of gangliosides was better understood, it was shown that tetanus toxin had an affinity for two particular gangliosides containing two sialic acid residues attached to the lactose component¹⁷ (Table 1).

Tetanus toxin does not seem to lose its toxicity as a result of being bound by ganglioside; nor does the ganglioside seem to be altered. If tetanus toxin (50 ng ml⁻¹) is incubated in the presence of gelatin for 1 h at 37° C with the gangliosides GGnSSLC and SGGnSSLC (300 µg ml⁻¹, molar ratio of ganglioside to toxin of 4 × 10⁵:1) there is no reduction in toxicity¹⁸. If 5 mg of mixed gangliosides (containing about 1 mg of GGnSSLC + SGGnSSLC) are injected intramuscularly into mice, however, followed 5 h later by an injection of 0.5 LD₅₀ of toxin in the same site, the mice are protected against the more severe degrees of tetanus, but in this case the molar ratio of ganglioside to toxin is much greater (3 × 10⁹:1) (ref. 19).

The interaction between tetanus toxin and ganglioside can be observed in two ways. First, a solution of tetanus toxin (5 mg ml⁻¹) and ganglioside (0.1 to 0.5 mg ml⁻¹) is centrifuged in an analytical ultracentrifuge. The complex of ganglioside and toxin will move rapidly down the centrifugal field, leaving some unbound toxin, the amount of which by difference from the original gives the amount bound¹⁸ (Table 1). Second, ganglioside is rendered insoluble by complexing it (as a mixed micelle?) with insoluble (and unreactive) cerebroside, and varying amounts of the complex are suspended in equal volumes of a solution containing about 1.5 ng toxin ml⁻¹ (about 45 LD₅₀ ml⁻¹). The insoluble ganglioside-cerebroside-toxin complex is spun down and the supernatant tested for toxicity in mice. In this way the least amount of ganglioside that will bind (say) 10 LD₅₀ of toxin can be determined. Not surprisingly, at these low concentrations of toxin far less toxin is bound per unit weight ganglioside than at concentrations 3 million times greater, but the differences between the toxin-binding capacities of different gangliosides are still evident (Table 1). The proportion of ganglioside in the insoluble complex is critical—thus complexes containing 25% ganglioside have a much greater capacity to bind tetanus toxin (and to protect mice against the toxin) than have complexes containing either 5 or 50% ganglioside⁹.

If relatively high concentrations of almost any ganglioside in solution are incubated for long periods with almost any toxin in the absence of a protective protein such as gelatin, the toxin will be inactivated. This nonspecific effect is of little interest, but has led to some confusion¹⁸.

So far, the specific interaction between tetanus toxin and the sialidase-labile gangliosides GGnSSLC and SGGnSSLC has not thrown any light on the mode of action of the toxin at the molecular level.

Serotonin

I have shown that mixed gangliosides in solution bound serotonin (5-hydroxytryptamine), tryptamine, lysergic acid diethyl amide, and ergometrine, but not adrenaline, nor-adrenaline, dopamine, histamine, or γ-aminobutyric acid; they also bound the convulsant drug strychnine and the related drugs brucine and thebaine¹⁷. I later found, however, that serotonin and strychnine were not bound by the ganglioside-cerebroside complexes that have such a great affinity for tetanus toxin⁹. The effect of ganglioside on serotonin has since been shown in other ways. Strips from rat stomach respond to serotonin, and Woolley showed that this response was diminished if the stomach strips were treated with sialidase, and restored if ganglioside was added to the enzyme-treated strips. Similar results were obtained with strips from ganglioside-deficient stomachs from rats reared on a galactose-rich diet²⁰. Gielen identified the serotonin-reactive ganglioside as the sialidase-labile SSLC (which, lacking the terminal galactose and the N-

acetyl/galactosamine residues, is unlikely to interact with tetanus toxin)²¹. But the intact cat erythrocyte contains SSLC, yet is incapable of binding serotonin. This is reminiscent of the observation mentioned above that mixed gangliosides in free solution bound serotonin, but not when complexed with cerebroside.

Cholera toxin

The actions of cholera and tetanus toxins could be considered similar in the sense that they both affect the passage of substances across cell membranes (those of intestinal epithelial cells and nerve cells respectively) without producing any morphological changes in their susceptible tissues. Cholera toxin has been isolated by Finkelstein as a protein with a molecular weight of 84,000 (ref. 22). In the final stages of purification of the toxin, it is possible to isolate an inert protein, choleraenoid (molecular weight 58,000), that is nearly, but not quite, immunologically identical with the toxin, and possibly derived from it²². When cholera toxin is introduced into the lumen of the small intestine, it causes an accumulation of fluid, leading to diarrhoea^{15,22}. When introduced intracutaneously it causes a local increase in capillary permeability²³. Indeed, the toxin acts on a wide variety of tissues and cells²², in which the effect usually appears to be mediated through the activation of adenylate cyclase. The subsequent increased levels of cyclic AMP in turn promote a wide variety of actions, including in the case of the intestinal epithelium, the secretion of chloride ions^{22,24}.

My collaborators and I have shown that homogenates of intestinal epithelium have a marked capacity to bind cholera toxin, whereas homogenates of liver, kidney, lung and heart seem to have no such capacity. Brain homogenate, however, proved even more effective than intestinal cells and it was then a simple step to show that mixed gangliosides were capable of binding the toxin, and were likely to account for the ability of these tissues to do so (brain contains about ten times as much ganglioside as intestinal cells)²⁵. Ganglioside not only binds cholera toxin, but, unlike the case with tetanus toxin, it also blocks its action in the small intestine, in the skin, and elsewhere. I then showed that of all the brain gangliosides, only the sialidase-stable monosialosyl ganglioside, GGnSLC (G_{M1}) had this effect^{26,27} (Table 1). The presence of a single sialic acid residue attached by a sialidase-labile bond, even to the terminal galactose residue, completely abolishes the capacity of the ganglioside to interact with cholera toxin. Also, treatment of intestinal epithelial cells with sialidase, an enzyme itself produced by the cholera bacillus, greatly increases their capacity to bind cholera toxin²⁶.

The blocking of the action of cholera toxin by added ganglioside indicates that when the toxin is bound to the extraneous ganglioside it is unable to attach itself to the receptor on the cell membrane, and therefore that the ganglioside GGnSLC may be very similar to, and even identical with, the membrane receptor of the toxin. GGnSLC also binds the biologically inert choleraenoid, and Pierce has shown that choleraenoid will block the action of cholera toxin in the small intestine, if placed in the intestine in advance of the toxin, one molecule of choleraenoid blocking one molecule of cholera toxin²⁸. King and I (unpublished data) have shown that one molecule of choleraenoid will block the capacity of ganglioside (in the form of a ganglioside-cerebroside complex) to block the activity of nearly one molecule of cholera toxin. These data suggest the possibility that choleraenoid may be the part of the cholera toxin molecule that is responsible for the binding of the toxin to the receptor on the cell membrane. This has been supported and amplified by Simon van Heyningen²⁹ who has shown that when cholera toxin is bound to an insoluble GGnSLC-cerebroside complex, a component, A (molecular weight about 22,000), may be eluted with neutral 8 M urea. In spite of

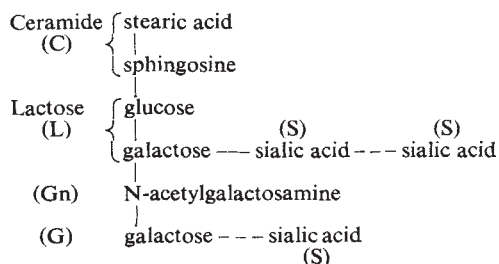
treatment with 8 M urea, this component has about 1% of the activity of the original toxin as tested in the rabbit skin. This activity is distinguished from that of the whole toxin by being unaffected by GGnSLC. The remaining portion of the toxin, in subunits B (molecular weight about 15,000), may be eluted from the ganglioside-cerebroside complex with neutral 6 M guanidine hydrochloride. Subunits B migrated with the same rate as subunits of cholera toxin on electrophoresis in polyacrylamide gel containing 6.25 M acid urea; after removal of the guanidine hydrochloride they were bound again by

Table 1 Interactions of the best-known gangliosides with tetanus and cholera toxins

Toxin	Molecules toxin bound per molecule ganglioside		
	Tetanus	Cholera	
Test	Ultracentrifuge	Toxicity	Toxicity
Toxin concentration	5 mg ml ⁻¹	1.5 ng ml ⁻¹	10 ng ml ⁻¹
Ganglioside*			
GnSLC (G _{M2})	< 0.005		< 0.000,5
GGnSLC (G _{M1})	0.04		0.35
SGGnSLC (G _{D1A})	0.04	0.000,05	< 0.000,5
GGnSSLC (G _{D1B})	0.28		< 0.000,5
SGGnSSLC (G _{T1})	0.28	0.000,6	< 0.000,5

The two sialic acid residues attached to the lactose component are necessary for optimal interaction with tetanus toxin, as is the terminal galactose residue; but the sialic acid residue attached to it seems to play no part. Only one ganglioside, which is sialidase-stable, interacts with cholera toxin, and the terminal galactose residue is again necessary for the interaction.

* It is convenient to use McCluer's abbreviated nomenclature for gangliosides³⁷ as it is self explanatory, and no less scientific than the widely used nomenclature of Svennerholm given in the brackets³⁸; thus, the trisialosyl ganglioside, sialosylgalactosyl-N-acetylgalactosaminylsialosylsialosyllactosylceramide (GT₁), becomes SGGnSSLc:



(Broken lines signify sialidase-labile bonds)

GGnSLC, and showed immunological identity with cholera-genoid. These data indicate that cholera toxin consists of two parts: a larger portion corresponding to cholera-genoid (composed of subunits B) which combines with the GGnSLC receptor on the cell membrane, and thus facilitates the entry of the toxin into the cell; and a smaller portion, component A, which does not combine with GGnSLC and is, somehow or another, responsible for the activity of the toxin within the cell. This is reminiscent of aspects of the composition and activity of diphtheria toxin^{30,31}, and of the toxic plant proteins, abrin³² and ricin³³.

The specific blocking action of cholera toxin by the ganglioside GGnSLC (G_{M1}) has subsequently been confirmed by Holmgren^{34,35} and by Cuatrecasas³⁶. Cuatrecasas has made the interesting observation that if exogenous GGnSLC is added to cells (lipocytes), and the excess ganglioside is washed from the cells, the capacity of these cells to bind and to react to subsequently added cholera toxin is increased. This observation is reminiscent of the effect, discussed in the previous section, of added exogenous ganglioside SSLC in restoring the reactivity to serotonin of ganglioside-deficient stomach strips. Cuatrecasas suggests that the added exogenous ganglioside is actually incorporated in the cell membrane, thus providing more receptor sites for the toxin. This is an interesting suggestion,

but there is as yet no direct evidence of incorporation of ganglioside, only increased binding of, and reactivity to, the toxin, and there may be other explanations. For example, added ganglioside might remove some substance blocking preexisting receptor sites (Gill, private communication).

Gill and King (unpublished data) have confirmed that added exogenous GGnSLC increases the response (measured in terms of adenylate cyclase activity) of intact pigeon red blood cells to cholera toxin, provided always that the amount of ganglioside is not in excess (otherwise the toxin would be blocked). If the red cells are first lysed with a bacterial haemolysin, the resulting ghost cells are still reactive to cholera toxin, but the reactivity is not increased by the addition of ganglioside. This indicates that if the toxin can enter the cell by some other means it does not need the entry by binding to the receptor on the outer surface of the cell membrane.

The accumulated evidence that the ganglioside GGnSLC is the receptor for cholera toxin is persuasive, and rekindles interest in the specific reaction between tetanus toxin and GGnSSLC (and SGnSSLC). Are these the receptors for tetanus toxin on the membranes of nerve cells? Or, as tetanus toxin has both a central and a peripheral action, and as botulinum toxin (which does not react specifically with ganglioside) has only a peripheral action (indistinguishable from that of tetanus toxin), is GGnSSLC responsible only for the central action of tetanus toxin?

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Joint transcription of two tRNA^{Tyr} genes from *Escherichia coli*

Alain Ghysen*

Departamento de Bioquímica, Facultad de Medicina, Universidad de Chile, Santiago, Chile and Medical Research Council Laboratory of Molecular Biology

Julio E. Celis†

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Biochemical evidence suggests that the two tRNA^{Tyr} genes of E. coli are transcribed together as a single precursor. A nuclease activity which cleaves incorrectly but specifically the tRNA^{Tyr} precursor has been revealed.

THE tRNA^{Tyr} species of *Escherichia coli* is coded for by two closely linked genes¹. In *sup3* mutants, one of these genes has a mutation in the anticodon which allows the tRNA to read UAG². *sup3*-Transducing phages have been constructed. One of them, referred to here as $\phi 80psup3$ (Kyoto), carries both the suppressor gene and the adjacent wild-type gene³. The other will be referred to as $\phi 80psup3$ (Cambridge), it carries only the suppressor gene as a result of an unequal recombination deleting the wild-type gene¹. Using $\phi 80psup3$ (Cambridge), Altman and Smith have shown that the single tRNA^{Tyr} gene is transcribed as a precursor containing 41 extra nucleotides at the 5' end and a few nucleotides at the 3' end of the mature tRNA sequence⁴. This precursor is then cleaved at the 5' end of the tRNA sequence by a specific endonuclease, RNase P⁵. The extra 3'-end nucleotides are subsequently removed by a different enzyme. The transcription and maturation scheme is not known in the normal situation where the two genes are present.

We recently reported the characterisation of mutations affecting the *sup3* gene carried by $\phi 80psup3$ (Kyoto)⁶; some of these result in greatly diminished synthesis of tRNA because of the low efficiency of correct processing of the tRNA precursor. Similar mutants in $\phi 80psup3$ (Cambridge) have previously been shown to reduce the yield of suppressor tRNA⁷. Two such mutants are A1 and A2. Other mutants such as U80, U81 and G82 do not appreciably alter biosynthesis of the mutant tRNA. Here we asked the question whether mutants A1 and A2 in the suppressor gene of $\phi 80psup3$ (Kyoto) also lower the amounts of tRNA synthesised by the neighbouring wild-type tRNA^{Tyr} gene. If the two genes are transcribed independently as separate precursor tRNAs, mutations in the suppressor gene would not be expected to alter the synthesis of the wild-type gene.

Our results suggest that the two tRNA genes are transcribed together as a common large precursor. We also show that in a RNaseP⁻ strain⁸ the tRNA^{Tyr} precursor is cleaved incorrectly by an specific nuclease.

Mutations in the *sup3* gene

The phage $\phi 80psup3$ (Kyoto) carries both the suppressor and the wild-type tRNA^{Tyr} genes. We have studied the expression of the two genes in cells infected with the original phage and with five mutants: A1, A2, U80, U81 and G82. These mutants have been previously described⁶, each contains

a single base change in the amino acid acceptor stem of the suppressor tRNA. The A1 mutation results in a G→A base substitution at the 5' end of the suppressor tRNA. Three independently isolated $\phi 80psup3$ A1 mutants (A1-16, A1-56 and A1-79) were compared with $\phi 80psup3$ (Kyoto) for the expression of the suppressor and the wild-type tRNA^{Tyr} genes. The total RNA synthesised after infection with the transducing phages was labelled with ³²P-orthophosphate and subjected to polyacrylamide gel electrophoresis⁹ which separates the total tRNA^{Tyr} from other tRNAs, most of which migrate as a broad band running ahead of the tRNA^{Tyr} band ('4S' tRNA).

The total tyrosine tRNA corresponds to a mixture of suppressor tRNA^{Tyr}, wild-type tRNA^{Tyr} and host tRNA^{Tyr}. The ratio (total tRNA^{Tyr})/('4S' tRNA) was calculated by direct counting of the two bands. The quantitative estimation of the various types of tRNA^{Tyr} was carried out as described in the legend of Table 1. The results are presented in Table 1, lines 1 to 4. Columns I to V show the ratio of total tRNA^{Tyr}

Table 1 tRNA^{Tyr} production in A1 infected cells

Mutant	Total tRNA ^{Tyr} —(%) Molar yield of the T ₁ fragments —'4S' tRNA					Hexanu- cleotide UCACAG	pAG	3' end* C81 U81	
	I	II	III	IV	V				
Single mutants									
A1-16†	29	30	30	31	37	0.12	0.09‡	—	—
A1-56†	28	27	29	ND	ND	0.11	0.10‡	—	—
A1-79†	25	24	37	ND	ND	0.12	0.08‡	—	—
<i>sup3</i>	107	ND	118	118	112	ND	—	—	—
Double mutants									
A1-16 U81	ND	ND	ND	103	118	0.017	0.40	0.35	0.32‡
A1-16 P2	ND	ND	ND	63	62	0.031	0.37	—	—
A1-56 P2	ND	ND	ND	60	65	0.036	0.33	—	—
Uninfected*	4	3	ND	ND	ND	0.55	—	—	—

tRNA^{Tyr} production in A1 infected cells. *E. coli* MB94 grown in low phosphate medium¹⁰ at 30° C was infected at a multiplicity of 30 and labelled for 60 min with ³²P-orthophosphate^{18,20}. The total RNA was subjected to polyacrylamide gel electrophoresis⁹. The ratio (total tRNA^{Tyr})/('4S' tRNA) was determined by direct counting of the two bands. The total tRNA^{Tyr} was extracted from the gels, digested with T₁ RNase and subjected to two-dimensional electrophoresis^{11,12}. Since all the mutants give characteristic T₁ fragments⁶, it is possible to determine the proportion of mutant and wild-type tRNA among the total tRNA^{Tyr}. Similarly, the proportion of host tRNA^{Tyr} can be determined since it contains the hexanucleotide UCACAG which is not present in tRNA^{Tyr} (ref. 10). Columns I to V show the ratio of the radioactivity present in the total tRNA^{Tyr} to that in the 4S tRNA in five experiments. The radioactivity of the various T₁ fragments was measured by liquid scintillation counting.

* The mutant 3' end has the C→U81 base substitution.

† A1-16, A1-56 and A1-79 are three independent isolated A1 mutants.

‡ Average result of two independent determinations.

§ The same results are obtained in uninfected cells or $\phi 80$ infected cells.

— Absence of the fragment.

ND Not determined.

*Present address: Division of Biology, California Institute of Technology, Pasadena, California 91109.

†To whom reprint requests should be addressed.

Table 2 Expression of the tRNA^{Tyr} genes

Mutant	4S tRNA	Coefficient of expression of the tRNA ^{Tyr} genes (%)		
		*Phage-coded tRNA ^{Tyr}	Wild-type gene†	Suppressor gene‡
Single mutants				
A1-16, A1-56, A1-79	25	0.23	0.20	0.02
<i>sup3</i>	109	1.0	0.5	0.5
Double mutants				
A1-16 U81	107	0.98	0.55	0.45
A1-16 P2, A1-56 P2	60	0.55	0.325	0.225

* The average ratio tyr tRNA/4S tRNA was calculated from the data in Table 1 and the background of host tyrosine tRNA has been subtracted.

† The full expression of both genes has been given value a of 1 (*sup3*).

‡ The values correspond to the ratio wild-type: suppressor found as result of the T₁ RNase digestion. The efficiency of transfer of the pAG fragment to the DEAE-cellulose paper is 90%; therefore a factor of 1.1 was applied to convert the molar yields of pAG into the ratios A1 tRNA: total tRNA^{Tyr}. The efficiency of transfer of the normal and modified 3' ends were assumed to be identical.

to the bulk of other tRNAs ('4S' tRNA) in five experiments. The last column shows the molar yield of the T₁ RNase fragments pAG (characteristic of the A1 tRNA) and UCACAG (characteristic of tRNA^{Tyr} (ref. 10)). The results of all three A1 mutants have been pooled in Table 2, column 1. In the second column the average ratio (total tRNA^{Tyr})/('4S' tRNA) is expressed as a function of the average ratio for $\phi 80psup3$ (Kyoto). This fraction represents the 'coefficient of expression' of the transduced tRNA^{Tyr} genes, equal to 1 in the case of *sup3* (Kyoto) where both genes are normally expressed. In the last two columns of Table 2 the 'coefficient of expression' is distributed between the wild-type and the suppressor genes according to the relative proportions of the two tRNAs revealed by T₁ RNase digestion. The results show that the A1 mutation reduces the expression of the adjacent wild-type gene to 40% of its normal value. This effect is *cis* specific since the amount of tRNA^{Tyr} is not reduced after infection by any of the A1 mutants. The A2 mutation (G→A change at the second position from the 5' end) reduces the amount of suppressor tRNA to 25-30% and the amount of wild-type tRNA to 70-75% of their normal value (data not shown). The U80, U81 and G82 mutants synthesise both tRNAs at normal levels (data not shown).

Reversion of the effect of A1 and A2 by secondary mutations

Double mutants showing an increased suppressor tRNA synthesis have been isolated from $\phi 80psup3$ A1 (Kyoto)⁶. One of them, A1U81, has a second base change at position 81, opposite to position 1 in the clover-leaf structure, while the second (A1P2) has no other change in the nucleotide sequence of the mature tRNA. One A1U81 and two independent A1P2 mutants were studied. The tRNA^{Tyr} production in infected cells are reported in Table 1, lines 5-7. The data has been treated as described above and summarised in Table 2. The U81 mutation raises the amount of the suppressor tRNA to almost normal levels. In addition U81 fully reverses the effect of the A1 mutation on the production of the wild-type tRNA. This indicates that the effects of the A1 mutation on the amounts of the suppressor and of the wild-type tRNAs are due to the same structural defect which is corrected by the second C→U81 base change. The P2 mutation also reverses the effect of A1 on both suppressor and wild-type tRNA production, however this reversion is only partial. P2 does not seem to be a promoter mutation since it induces different increases in the coefficient of expression of the two tRNAs. Therefore P2 is most likely a precursor mutation which somehow compensates for the structural defect caused by the A1 mutation. A double mutant, A2U80, obtained from $\phi 80psup3$

A2(Kyoto)⁶ has also been studied. The secondary C→U mutation at position 80 completely reverses the effect of A2 on the synthesis of the suppressor and the wild-type tRNAs (data not shown).

tRNA^{Tyr} precursors in RNase P⁻ cells

To characterise the transcription products of the phage-transduced tRNA^{Tyr} genes we used the temperature-sensitive RNase P⁻ mutant described by Schedl and Primakoff.⁸ At 42° C the tRNA-processing enzyme RNase P is inactive, thus allowing the isolation of tRNA precursors in which the 5' termini has not been removed. We analysed the RNAs

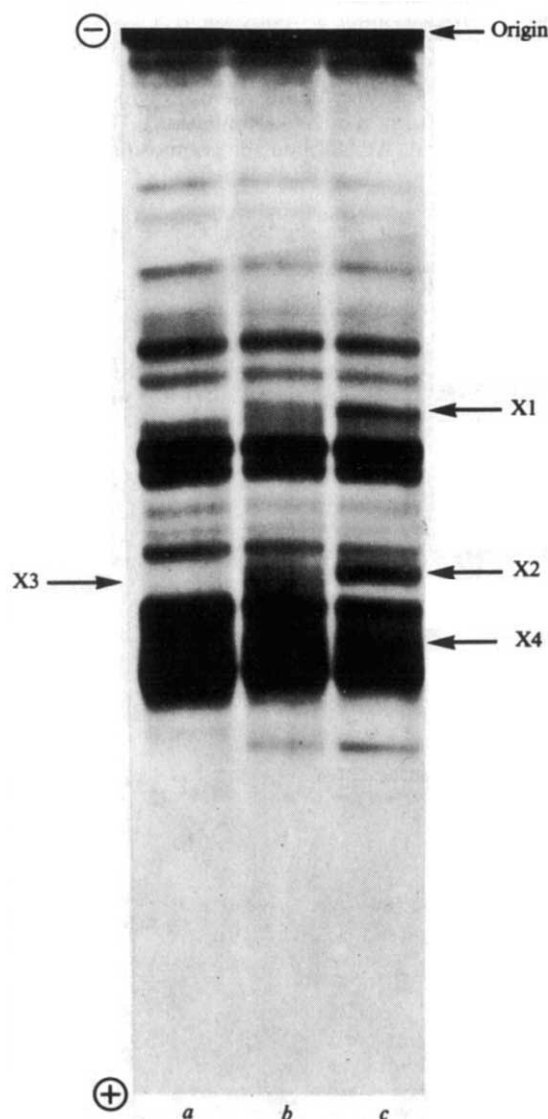


Fig. 1 Polyacrylamide gel electrophoresis of ³²P-RNAs from A49 infected cells. A49 cells (RNase P⁻, kindly provided by P. Schedl) grown at 30° C in TPGA medium²¹ (5 × 10⁻⁴ M phosphate) to 4 × 10⁸ cells ml⁻¹ were collected by centrifugation and re-suspended in one-fifth volume of TPGA medium (5 × 10⁻⁵ M phosphate) prewarmed at 42° C. After 10 min of aeration at 42° C, 0.02 vol of M MgSO₄ was added and 0.5 ml of cells were infected at a multiplicity of 30. After 10 min adsorption without aeration the cells were diluted 10-fold into TPGA medium (5 × 10⁻⁵ M phosphate) prewarmed at 42° C (*t* = 0). 5 mCi of ³²P-orthophosphate were added at *t* = 27 min and the incorporation stopped 10 min later by adding an equal volume of buffer saturated phenol prewarmed at 42° C. This procedure is similar to that described by Schedl and Primakoff⁸. Radioactive samples were applied to 12% polyacrylamide gels as described by Peacock and Dingman.⁹ a, $\phi 80$; b, $\phi 80 psup 3$ A1U81 (Kyoto) and c, $\phi 80 psup 3$ (Cambridge).

synthesised in RNase P deficient cells infected with $\phi 80psup3$ (Kyoto), $\phi 80psup3$ (Cambridge) and various mutants. Figure 1 shows an autoradiograph of a polyacrylamide gel electrophoresis of ^{32}P -RNAs from RNase P⁻ cells infected with (a) $\phi 80$, (b) $\phi 80psup3$ A1U81 (Kyoto) and (c) $\phi 80psup3$ (Cambridge) and labelled with ^{32}P -orthophosphate at 42° C. Bands X1, X2, X3 and X4 are absent from cells infected with $\phi 80$ and all contain tRNA^{Tyr} sequences. The pattern of RNAs synthesised after infection with $\phi 80psup3$ (Kyoto) is identical to that from $\phi 80psup3$ A1U81 (Kyoto). All the bands were extracted from the gels, digested with RNase T₁ and fingerprinted^{11,12}. The results for A1U81 are shown in Fig. 2. X1 corresponds to the precursor tRNA described by Altman and Smith⁴. It contains the sequence of precursor A1U81 *sup3* tRNA, however, and not that of the wild-type tRNA^{Tyr} precursor (Fig. 1a). The 3'-end fragment of this precursor was shown by pancreatic RNase digestion to contain the C→U81 base change. It also contains a few additional 3' nucleotides (including the dinucleotide AU) which are not found in the mature 3'-end fragment. The UAG fragment results from the G→A mutation. The absence of C81-modified 3' end, ACUG and the composition of the anti-

codon indicate that no wild-type tRNA sequence is present in this RNA band.

Band X2 (Fig. 2b) corresponds to a 1:1 mixture of A1U81 and wild-type sequences as judged by the quantitation of the two 3'-end fragments (containing U81 and C81 respectively), ACUG and the anticodon fragment. None of the fragments of the precursor 5' end can be detected, yet these tRNAs differ from the mature tRNAs in the absence of the normal T₁ 5'-end fragments (pAG and pGp respectively). In the case of the suppressor tRNA pAG is replaced by a new 5'-end fragment, UAG. This fragment results from the incorrect cleavage of the precursor between residues 40 and 41 (ref. 4) as shown in Fig. 3. Also, pancreatic RNase digestion of band X2 tRNA gives AGU and no pAGU can be detected. The situation however is not clear for the wild-type tRNA^{Tyr} since we do not find any new fragment that replaces pGp. The 3' end of both tRNAs contain some or all of the 3'-end extra nucleotides present at the 3' end of band X1 RNA.

Band X3 (Fig. 2c) corresponds to a 1:2 mixture of A1U81 and wild-type sequences; no other difference could be detected between the fingerprints of bands X2 and X3. These bands do not always separate; the occasional separation could conceivably be due to a partial loss of some of the 3'-end extra nucleotides.

Band X4 RNA (fingerprint not shown) differs from band X2 and X3 by having mature 3'-end fragments. Since this band is heavily contaminated and the fingerprints contain pGp it is difficult to assess whether the wild-type tRNA has an abnormal 5' end. In the case of the suppressor gene we can detect small amounts of pAG, however this fragment is not in molar yields with the U81 3'-end fragment. It is likely then that at least the suppressor tRNA contains an abnormal 5' end.

Although we analysed all the other bands in the gel in Fig. 1b, we have been unable to find a precursor for the wild-type tRNA or a doublet precursor. Further evidence that a singlet precursor for the wild-type tRNA gene cannot be detected has been obtained by analysing the RNAs synthesised in RNase P⁻ cells infected with A1 mutants. Infection with $\phi 80psup3$ A1 (Cambridge), which carries only the A1 tRNA gene, yields none of the 1 to 4 bands as a result of the thermosensitivity of the A1 mutation. When the infecting phage is $\phi 80psup3$ A1P2 (Kyoto), which carries both the A1 and the wild-type genes, only bands X3 and X4 can be detected (results not shown). T₁ RNase digests of band X3 (Fig. 2d) and X4 (not shown) confirm that they contain the wild type but not the A1 sequence. Similar analysis of the $\phi 80psup3$ - phage (Cambridge) which carries only the wild-type gene yields bands X1, a diffuse band in the position of X2 and X3 and band X4 (results not shown).

Common tRNA precursor

Some mutations, which break base pairs in the amino acid acceptor stem of the suppressor tRNA^{Tyr} decrease the yield of tRNA coded by the adjacent wild-type gene. This result suggests that the two tRNAs are synthesised as a single transcription product ('doublet' precursor), so that a structural deformation close to the RNase P cleavage point affects the processing, and therefore the yield of both tRNAs. This explanation is further supported by the observation that no 'singlet' precursor can be detected for the wild-type tRNA^{Tyr}. We feel that the origin of the tyrosine tRNAs sequence found in RNase P⁻ cells is best explained as pictured in Fig. 3. The two genes are transcribed as a long precursor containing both sequences, the suppressor sequence being first from the 5' end. The length of the spacer between the two tRNAs cannot be derived from our data, it could be 35-70 nucleotides¹³. As a result of endonuclease cleavage(s), this unstable precursor yields a singlet precursor containing the suppressor sequence (band X1), and a wild-type tRNA with immature 5' and 3' ends (bands X2 and X3). The 'singlet' precursor is further cleaved incorrectly but specifically between residues 40 and 41 of the

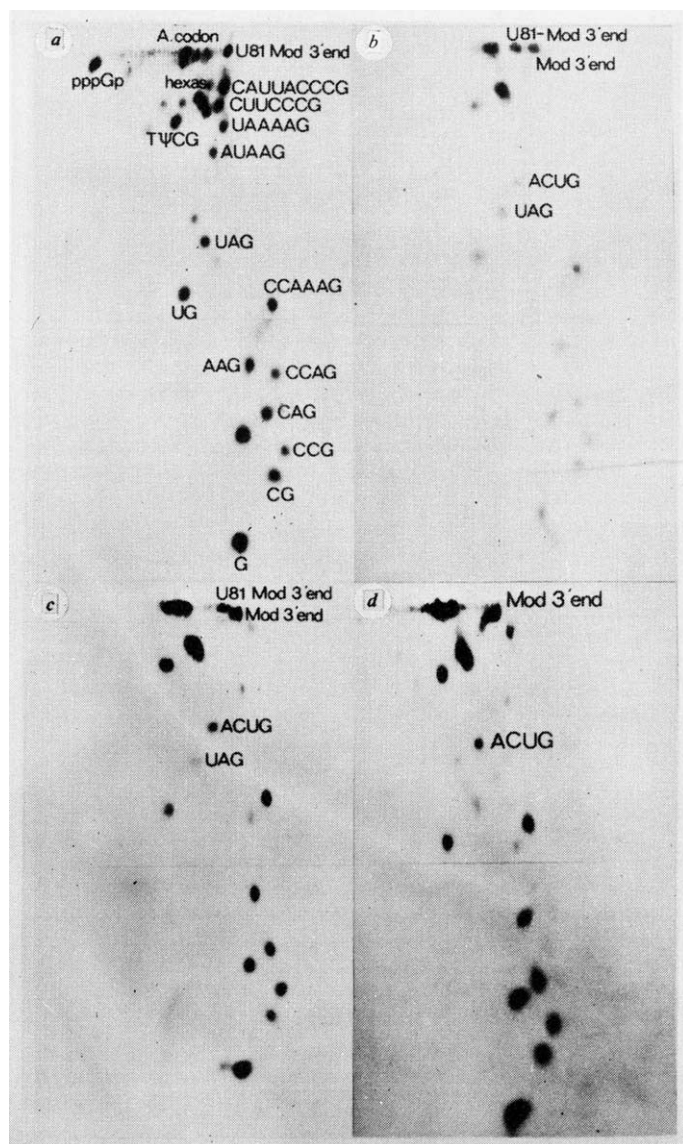


Fig. 2 Two-dimensional fractionation of RNase T₁ digestion products. a, A1U81 band X1; b, A1U81 band X2; c, A1U81 band X3 and d, A155P2 band X3. Electrophoresis on cellulose acetate in pyridine acetate, 7 M urea, pH 3.5 right to left; and on DEAE paper in 7% formic acid (v/v) from top to bottom. U81-Mod 3' end=3'-end fragment containing the U81 mutation and the extra 3'-end nucleotides. Mod 3' end=3'-end fragment containing the 3'-end extra nucleotides.

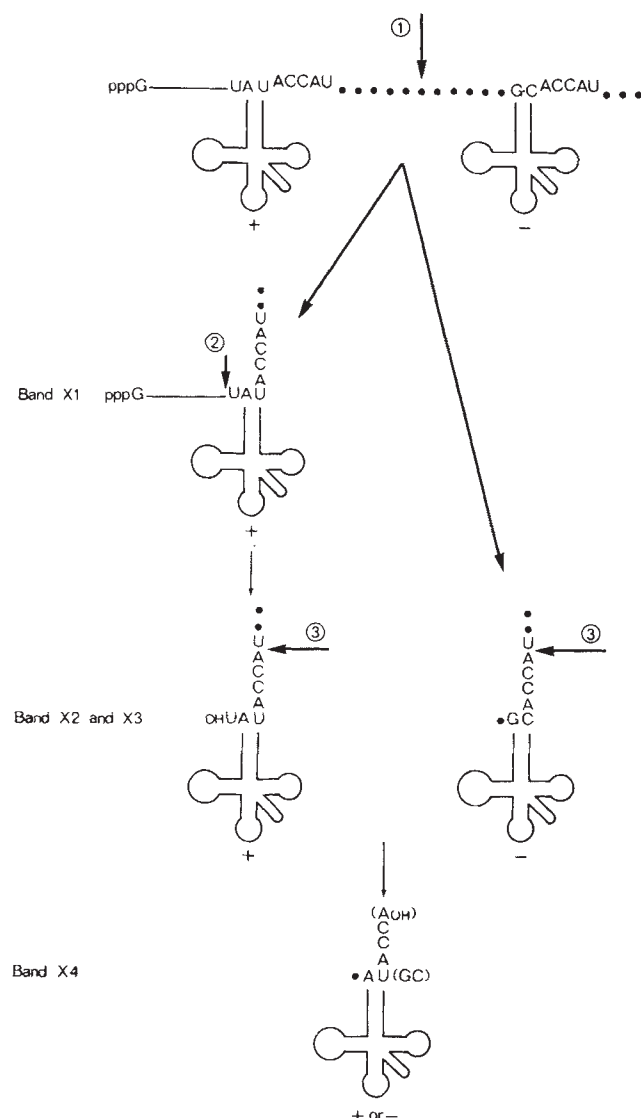


Fig. 3 Possible origin of the precursor cleavage products. The first tRNA sequence of the doublet precursor corresponds to the A1U81 suppressor, the second is the wild-type sequence. The black dots indicate that neither the length nor the sequence of these fragments is known. The arrows indicate the proposed nuclease cleavage. The last residue of the 'mature' tRNA has been written as (A_{OH}) since we have not established its identity. + and - correspond to the suppressor and wild-type anticodon respectively.

precursor fragment⁴ (arrow 2 in Fig. 3) to give a suppressor tRNA beginning with HOUAG ---- at the 5' end and with

immature 3' end. At present we do not know whether the enzyme that cleaves incorrectly at the 5' end of the suppressor tRNA is also responsible for the cleavage of the initial doublet precursor at the 5' end of the wild-type tRNA. Finally, the extra 3' end nucleotides are cleaved (arrow 3 in Fig. 3). At least in the case of the suppressor tRNA the 5' end seems to remain abnormal.

Another example of common precursor for closely linked tRNA genes has been reported in the case of the T4 coded tRNAs^{14,15}. Furthermore the occurrence of other closely linked tRNA genes in the bacterial genome also raises the possibility of large bacterial tRNA precursors containing two or more tRNA sequences¹⁶⁻¹⁸. Indeed, Schedl and Primakoff⁸ have recently reported that they have found tRNA precursors containing more than one tRNA sequence in RNase P⁺ cells.

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Simian virus 40 in a human cancer

Federico Soriano, Charles E. Shelburne & Muharrem Gökçen

Research and Education Division, Health Central Inc. & Life Sciences Foundation, Minneapolis, Minnesota 55422

Simian virus 40 (SV40) and SV40 T (tumour) antigen have been detected in the malignant melanoma metastases of a patient in which specific viral and T antibodies were demonstrated.

THE patient in our investigation, a plumber born in 1894, had no personal or familial history of chronic illness or congenital anomaly. A pigmented skin growth in his upper back, which he had noticed 2-3 yr before, was removed in 1964 and found to be a malignant melanoma. The diagnosis

was later confirmed in biopsies of axillary and cervical lymph node metastases, in the cells of a pleural exudate that developed shortly before death, and at autopsy, performed in December 1967. No history of immunisation with vaccines likely to contain SV40 was later found. At autopsy there were widespread pigmented or unpigmented metastases in the skin and muscles of the shoulder and chest wall, in the lungs, pleuras, diaphragm, liver, lymph nodes, dura, brain and heart, also hydrothorax and bronchopneumonia.

The medium used for tissue culture consisted of equal parts of McCoy's 5a medium and medium 199, supplemented with foetal bovine serum and antibiotics. Metastatic and normal tissues taken at autopsy were cultured after mincing or trypsinising. The tumour cultures initially contained tumour cells, fibroblasts and some lymphocytes. All cultures were kept for 1–3 months but were gradually lost. Only cultures of normal spleen and normal kidney were frozen for future subculture; none of the others showed adequate growth. Continuous cultures of typical lymphoblastoid cells forming floating colonies eventually emerged from cultures of the spleen and of muscle and lymph node metastases, however. These three exclusively lymphoblastoid cell lines have been cultured for several years.

Virus studies

Several specimens of lung, liver and muscle metastases and of normal spleen and kidney had been frozen since autopsy at -94°C without medium or cryoprotective agents. Beginning 14 months after autopsy, the specimens were thawed, minced, forced through fine sieves while rinsing, centrifuged, and resuspended at 20% concentration in medium containing 10% heated foetal bovine serum. Aliquots were frozen for future use. The suspensions consisted of broken cells and debris. They were centrifuged at 500g for 10 min and each of several monolayers grown in 30-ml flasks was inoculated with 0.1 ml of the pellet. The cultures were maintained with medium containing heated foetal bovine serum and examined regularly with an inverted phase contrast microscope.

In the first isolation experiment with various human and animal cell cultures, signs of cytopathic effects characteristic of SV40 were first observed after 30 d in one primary grivet (African green monkey) kidney culture inoculated with a lung metastasis specimen. Ten days later virus was collected (isolate A). All cultures had been observed for more than 1 month before inoculation and were negative for virus and *Mycoplasma*. All negative and control cultures were held for 6 weeks after virus collection; no virus growth occurred.

To avoid the risk of viral contamination in new lots, primary grivet kidney cultures were permanently discontinued after this experiment; primary rhesus kidney cultures were never used. Subsequent isolations were carried out with the grivet kidney-derived BSC-1 cell line; cell stock was obtained from the American Type Culture Collection. From another lung metastasis virus was recovered in three further successive attempts (isolates B, C and D). In two other separate experiments virus was isolated from liver and muscle metastases (isolates E and F). In no case was any incipient cytopathic effect characteristic of SV40 observed sooner than the fourth week of incubation or later than the fifth. In each experiment all negative cultures and controls were kept, without showing cytopathic effects, for at least 8 weeks after virus collection from the positive cultures. Over the years during which these isolations were made no viral or mycoplasmal contamination was detected in several thousand stock and control cultures monitored periodically by electron microscopy or cultural methods.

Several other attempts with the previously positive specimens or with different tumour specimens were unsuccessful. Virus was recovered from about 3% of approximately 500 cultures inoculated with original tumour tissue pellets or with blind passages. In the successful isolations, excluding the other attempts, a total of 11 ml of packed tissues (0.1 ml per culture) yielded 15 positive cultures. It can be estimated that approximately 1 infectious unit was contained in 1 g of the pellets or in no less than 1 g of intact tumour at the time of death, and that virus was detectable only in some parts of the tumours.

In further experiments inoculation of tumour pellets into less sensitive grivet kidney cell lines (CV-1, Vero) yielded no virus and BSC-1 cultures remained negative after inoculation with cell-free tumour extracts. No virus was recovered from BSC-1 cells inoculated with pellets of the normal spleen and normal kidney, nor from human fibroblast and primary embryonic kidney cultures inoculated with pellets of the tumours or of the normal organs. No virus grew, and no viral or T antigen was detected by immunofluorescence, in BSC-1 cultures inoculated with live (cocultivation) or homogenised cells of the three lymphoblastoid lines. Each of these experiments lasted 2–3 months and was repeated at least once.

Electron microscopy of a brain metastasis that had been kept in formalin revealed no virions in several samples of the tumour and of the adjacent white matter. There was good preservation of fine detail.

Mice and Syrian hamsters were inoculated at birth with pellets of the normal and metastatic tissues and of pleural tumour cells obtained before death. No meaningful disease occurred during the natural life span of the survivors.

In cross-neutralisation tests no significant differences were found between the six isolates and two known SV40 strains. Strain J436 and baboon antiserum to J436 were obtained from the National Institutes of Health, strain A2895 from the American Type Culture Collection; rabbit antisera to A2895 and to the six isolates were produced in our laboratory. The two known antisera neutralised the six isolates and two known strains to the same extent; antiserum to each isolate had about the same titre against the particular isolate as against the two known strains. Since each isolate was also proved to be oncogenic for newborn hamsters and no difference from SV40 was found in further work, we have designated isolates A to F collectively as Strain F of SV40, or SF.

Strain F is identical to other strains in its intrinsic properties, growth in cell cultures, cytopathic effects, cell-transforming efficiency, oncogenicity, and electron microscopic features^{1–4}. Subcutaneous or intracerebral inoculation of newborn hamsters induced local sarcomas or ependymomas as described for SV40 (refs 5 and 6). Cultures of non-tumour human cells supported its growth and underwent malignant transformation. Inoculation of SF-transformed mouse or hamster cell cultures in the respective hosts resulted in malignant tumours. As within SV40 strains, tumours can be prevented in hamsters inoculated at birth with strain A2895 by injecting SF during the incubation period; conversely, SF tumours can be prevented by a second injection with A2895.

Identity of the cellular T antigen induced by SV40 (ref. 7) with the SF-induced T antigen was demonstrated by indirect immunofluorescence⁸. Coverslip cell cultures fixed in acetone were used immediately or stored at -30°C . Known SV40 T-positive cultures were TT-101, a virus-free SV40 hamster tumour cell line (Flow Laboratories, Rockville, Maryland) and BSC-1 cells inoculated with A2895; SF T-positive cultures were S-419, a virus-free line derived from an SF hamster tumour, and BSC-1 cells inoculated with SF. Infected BSC-1 cells were fixed 3 d after inoculation. Control cultures were normal continuous (BHK-21) or primary hamster kidney cells, and normal BSC-1 cells. Known SV40

T antiserum was obtained from Flow Laboratories and from hamsters bearing transplanted A2895 tumours, SF T antiserum from hamsters with transplanted SF tumours, and control serum from normal hamsters; all T antisera were free of viral antibodies. Fluorescein-labelled γ -globulin against hamster γ -globulin was obtained commercially. The SV40 T antisera stained SV40 T-positive and SF T-positive cells to the same extent, and the same was true for the SF T antiserum; all controls were negative. Each antiserum was absorbed equally well with T antigen extracts from hamster tumours induced by known SV40 or SF.

Antibody and antigen studies

Patient's serum was unavailable, but several specimens of pleural exudate had been stored at -30°C . Early attempts to demonstrate antibodies in exudate obtained shortly before death were unsuccessful. The patient's blood belonged to group O, however, so a search for samples with at least agglutinating antibodies for AB erythrocytes was made. Only the earliest sample, stored 6 d before death, had haemagglutinating activity. It contained IgG, IgA, IgM, and detectable SV40 viral and T antibodies.

Heat-inactivated exudate, diluted 1:2, neutralised 70–100 median tissue culture infectious doses of SF, A2895 or J436 in BSC-1 cells observed over 3 weeks but it did not do so when diluted 1:2 in a concentrated rabbit γ -globulin of a high titre against human γ -globulin, thus indicating activity caused by antibody. The antihuman γ -globulin alone had no effect on the viruses or the cells.

T antibodies were demonstrated as described above by indirect immunofluorescence with known SV40 T-positive cultures (virus-free TT101 cells and A2895-inoculated BSC-1 cells), SF T-positive cultures (virus-free S-419 cells and SF-inoculated BSC-1 cells) and controls. To prevent virus growth, inoculated BSC-1 cultures were maintained during the 3 d of incubation with daily changes of medium containing $20\text{ }\mu\text{g ml}^{-1}$ 5-fluorodeoxyuridine and $20\text{ }\mu\text{g ml}^{-1}$ 1- β -D-arabinofuranosylcytosine. Besides normal BSC-1 and hamster cells, controls included previously T-positive coverslips in which the antigen had been destroyed by storing for several weeks at 4°C and positive controls stained with hamster T antiserum and labelled anti-hamster γ -globulin. The exudate was treated with heparin to inhibit any solubilisation of T antigen by RNase it may have contained, and with thimerosal and phenol to inhibit microbial growth. Normal human serum and the labelled globulin applied alone served as controls. The exudate stained typically and exclusively the nuclei of all T-positive cells. Six applications, each followed by 1 d of incubation at 4°C , were needed for best results with the tumour cells. Quicker results were obtained with inoculated BSC-1 cells, which produce more antigen, and with exudate concentrated by ammonium sulphate precipitation. Concentrated exudate absorbed with T antigen extracted from BSC-1 cells infected with SF or A2895 lost its staining activity, but it did not when absorbed with the same extracts previously exposed—after sterilising by filtration—to 'warm' temperatures, that is, to 4°C for several weeks or 22°C for a few days, or when absorbed with normal BSC-1 extracts. Concentrated exudate blocked the binding of fluorescein-labelled hamster T antiserum to T-positive BSC-1 cells, as judged by a conspicuous reduction of the staining intensity. Normal human serum and high titre anti-nuclear sera from patients with systemic lupus erythematosus had no effect.

T antigen was demonstrated in the patient's tumour tissue suspensions. These were sonicated, treated with RNase and centrifuged. The antigen was precipitated from the supernatants with saturated ammonium sulphate (32%) or

by acidifying to pH 5 with 0.2 M acetic acid⁹. The precipitated antigen was redissolved and dialysed in phosphate-buffered saline, pH 7.2. With the small amounts involved little final concentration—but some purification—was achieved. For positive control, an SF hamster tumour that had been kept frozen was extracted in the same way after homogenising. These extracts served also as negative controls after exposure to warm temperatures to destroy the antigen, as described above (inactivated T antigens). Similar extracts from a human placenta and a human myoma were additional control antigens.

A solid-phase microradioimmunoassay¹⁰ was adapted to demonstrate T antigen. Serial two-fold dilutions of antigens in phosphate-buffered saline were placed in wells of microtitre plates, 25 μl per well, and dried at 4°C under vacuum in a desiccator containing calcium sulphate. After methanol

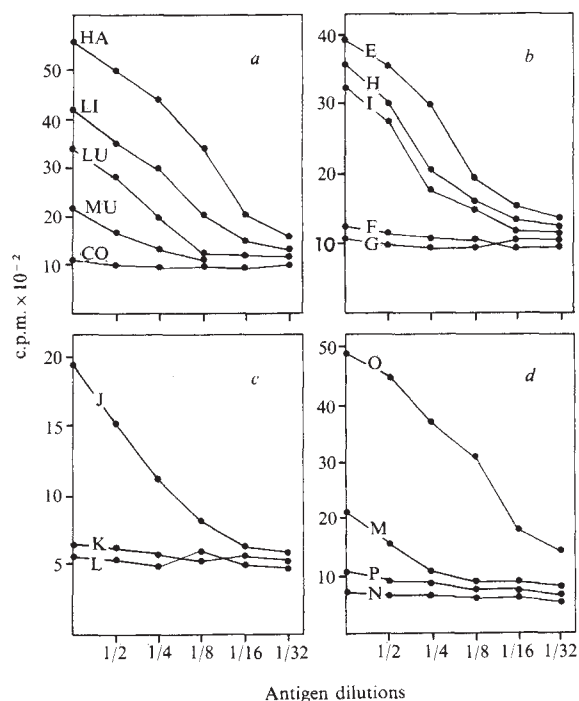


Fig. 1 Radioimmunoassays of the patient's T antigen and antibodies. *a*, Liver (LI), lung (LU) and muscle (MU) tumour antigen extracts; a negative control (CO); an SV40 hamster tumour extract (HA) as a positive control. All reacted with hamster T antiserum and anti-hamster ^{125}I - γ -globulin. The following tests were performed with the patient's liver tumour antigen with a titre of 16. *b*, Absorptions: hamster T antiserum, unabsorbed (E) or absorbed with the patient's T antigen (F), hamster T antigen (G), inactivated patient's T antigen (H) or placenta antigen (I), followed by anti-hamster ^{125}I - γ -globulin. *c*, Antibodies: patient's exudate (J), normal human serum (K) or systemic lupus erythematosus serum (L) followed by anti-human ^{125}I - γ -globulin. *d*, Blocking tests: normal hamster serum (M) or hamster T antiserum (N), both followed by exudate and anti-human ^{125}I - γ -globulin; normal human serum (O) or exudate (P), both followed by hamster T antiserum and antihamster ^{125}I - γ -globulin. (For details see text.)

fixation the plates were rinsed with buffered saline, and 20 μl per well of each appropriate serum or γ -globulin were successively applied for 1 h at 22°C . The plates were rinsed after each step; the final γ -globulin against hamster or human γ -globulin was labelled with ^{125}I (^{125}I - γ -globulin). The wells were then cut out, placed in tubes and counted for radioactivity in a γ spectrometer. The average c.p.m. bound to triplicate test wells divided by the average c.p.m. bound to control wells gave the binding ratio, ratios of 1.5 or greater

being taken as significant. The titre was the reciprocal of the highest dilution giving significant binding.

The T antigen titres obtained with hamster T antiserum and anti-hamster ^{125}I - γ -globulin in the extracts of the patient's metastases and of the hamster tumour are shown in Fig. 1a. The differences in titre are considered irrelevant. The suspensions of metastases from which the extracts were made had been thawed and rewarmed several times for various periods of time; the hamster tumour had never been used; the muscle specimen contained many normal muscle fibres. The placenta and myoma extracts and the inactivated T antigens gave approximately similar results, no different from the background given by the labelled globulin alone; a representative curve is shown. The following assays were performed with the liver tumour extract with a titre of 16 (patient's T antigen). T antibodies were removed from the hamster T antiserum by absorption with patient's or hamster T antigen but not with inactivated patient's T antigen or placenta extract (Fig. 1b). The specificity of the patient's T antigen and antibodies was further demonstrated. The patient's exudate bound significantly to the patient's T antigen but normal human serum or high titre anti-nuclear serum from patients with systemic lupus erythematosus did not (Fig. 1c). In blocking tests, hamster T antiserum and exudate inhibited the binding of each other to the patient's T antigen but lupus and normal human serum or normal hamster serum had no effect (Fig. 1d).

No viral or T antigen was detected by this method or by immunofluorescence in the three lymphoblastoid cell lines derived from metastatic tissues and from the normal spleen, nor by immunofluorescence in subcultures of the normal renal epithelium or of the splenic mixture of fibroblasts, macrophages and lymphoid cells.

SV40 in humans

The findings in this case correspond to those in hamsters with SV40 tumours. Virus and T antigen were detected in lung, liver and muscle metastases but not in the normal spleen and kidney or in the three lymphoblastoid cell lines; viral and T antibodies could still be detected in the dying patient.

Not much is known about SV40 multiplication in humans. It has rarely been isolated: from faeces after ingestion¹¹, from throat swabs after respiratory route inoculation¹², and from the brains of two patients with progressive multifocal leukoencephalopathy¹³. Only human cells are truly semipermissive *in vitro*, both undergoing transformation and permitting SV40 growth. Normally, monkey cells are only permissive, and hamster or other animal cells are nonpermissive, that is, can only be transformed. Although humans are apparently inefficient hosts¹⁴, initial *in vivo* growth, sufficient to induce viral (but not anti-T) antibody, is inferred from the titres in persons infected by the respiratory¹² or subcutaneous¹⁵ route, in monkey handlers and in persons (including some cancer patients) with no known SV40 contact¹⁴; this last group suggests a natural infection from an unknown reservoir and by an unknown route¹⁴.

The minute amounts of SV40 detected exclusively in the patient's tumours could not alone account for a viral antibody response, and do not suggest virus growth but the well known occasional release of minimal residual infectivity by some nonlysing malignant cells. Moreover, since no differences in susceptibility of any human cells for SV40 infection are known, it is very unlikely that this tumorigenic virus could be fortuitously present in three different tumorous organs but not in the normal ones.

The presence of readily detectable T antigen in the tumour extracts cannot be explained by local virus growth.

In infected monkey organs SV40 proliferates optimally but individual T-containing cells could not be detected¹⁶. As well as high anti-viral titres the animals can develop T antibody¹⁶, so the innocuously infected monkey tissues must release immunogenic but *in vivo* probably undetectable amounts of T antigen. By contrast, T antibodies have not been detected in noncancerous persons with high and persistent anti-viral titres^{14,15,17}. Some reactivity with SV40 T antigen has been detected in only three cancer-free persons who had insignificant or no SV40 anti-viral activity; it must be considered to be caused by cross-reacting antibodies directed against an antigen produced by BK virus, a different agent¹⁷. The failure to detect T antibody in many cancer patients¹⁴ may indicate that there are indeed very few SV40 tumours, or that therapy prevented the tumours becoming very large and partly necrotic and releasing the intranuclear antigen¹⁸. Failure may also be a consequence of the fact that the antibody levels present may be detectable only by more sensitive techniques, or of immunosuppression by anticancer drugs or irradiation.

We have not detected T antigen in other melanomas; the evidence does not imply any obligate association of human malignant melanomas with SV40. Injected intravenously, SV40 can cause leukaemia, lymphosarcoma, reticulum cell sarcoma, osteogenic sarcoma and possibly other malignancies in hamsters¹⁸. Viral antibodies, and by inference SV40 infection, occur in childhood¹⁴. Human SV40 malignancies may conceivably¹⁹ develop only occasionally after a very long time or fortuitous triggering. The possibility of SV40 hybridising *in vivo* or *in vitro* with other viruses must be considered, for hybrids could spread the tumour-inducing genes of SV40 more efficiently than the pure virus; the adenovirus-SV40 hybrids that have originated *in vitro* are well known.

SV40 tumour cells, like all other cells permanently transformed by SV40, contain without exception specific T antigen, a nonviral marker of unknown function that indicates integration of the viral into the cellular genome^{7,19}. No systematic search for SV40 T antigen in human cancers has been made. Confirmation of the oncogenicity of SV40 for humans will have to come from finding additional cases, especially by T antigen detection.

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Sequence § (*D. salexigens*) is tentative and incomplete (R. P. Ambler, M. Bruschi and J. Le Gall, unpublished observations). The one-letter notation used is that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature¹⁹. The haem-binding sites are underlined. Residues common to each sequence are shown in capitals. This choice is somewhat subjective, as deletions can be juggled to improve the apparent match.

Oxidation-reduction states

By adding aliquots of sodium dithionite, the reduction at 25° C of the cytochrome c_3 from *Desulfovibrio vulgaris*, prepared as described previously⁶, has been studied by following the paramagnetically shifted NMR resonances⁷ which lie to very low field (Fig. 1). The number of methyl resonances in this region indicates the number of oxidised haem groups present. By monitoring selected peaks the disappearance and appearance of three different species can be followed readily during a titration (Fig. 2). The titration shows that there must be four haems per molecule which are reduced in two two-electron steps separated in midpoint potential by about 50 mV.

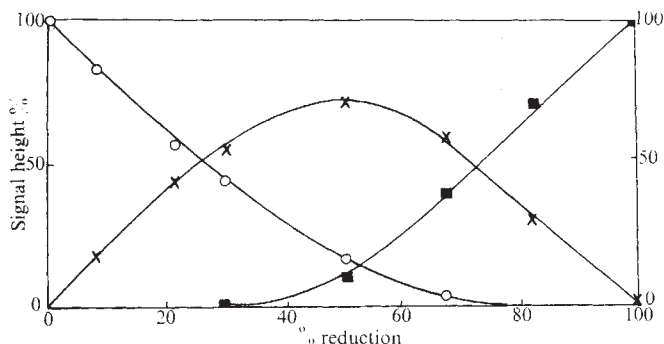


Fig. 1 Appearance and disappearance of NMR lines associated with the three different redox states of cytochrome c_3 . O, Concentration of the fully oxidised state which has been followed by measuring the area of several contact shifted methyl resonances at very low field; x, concentration of the intermediate form, followed by the appearance and disappearance of the new contact shifted methyl resonances on addition of reducing agent, sodium dithionite; ■, the appearance of the diamagnetic form, followed by the appearance of the single proton resonances at very low field attributed to two histidines.

EPR measurements have shown quite independently that the four haems are quite distinct⁸. The NMR reduction-titration (Fig. 2) starts from four distinct and separate paramagnetic haem species, (12-16 low-field methyl resonances, shown more clearly in Fig. 3) has an intermediate of two paramagnetic species (seven or eight low-field methyl resonances) and then gives a fully reduced form of four diamagnetic haem species (no low-field methyl resonances). The reaction is fully reversible and addition of oxygen to the fully reduced species gives an unaltered fully oxidised spectrum. Before discussing the NMR spectra further it is necessary to characterise the spin states of the haems.

The absorption⁹, the NMR (ref. 10) and the ESR (ref. 8) spectra of cytochrome c_3 from *Desulfovibrio vulgaris* are all characteristic of low-spin Fe(II) and Fe(III) in all oxidation states of the protein. The four cysteine sequences already described as well as the absorption spectra indicate that all the haems are bound through thioether linkages (normal cytochrome c links) and thus that one of the iron bound residues of each haem is a histidine of the sequences Cys-x-x-Cys-His or Cys-x-x-x-Cys-His. Now as each iron of each haem is low spin it is required to be bound to one other strong ligand which therefore cannot be water or carboxylate, but could be methionine, lysine or histidine. From the g values of the EPR spectra of the fully oxidised and half oxidised protein⁸, the other ligands are probably nitrogen ligands, that is, either histidine or lysine. The NMR spectrum of the oxidised protein also rules out methionine as a haem ligand, that is either histidine or lysine. The NMR spectrum resonance around +20 p.p.m. (as found in cytochrome c)⁷ and the absorption spectrum confirms this as there is no 695 nm band typical of methionine binding⁹. McDonald and

Phillips¹⁰ have pointed out that the NMR spectrum also excludes lysine as a haem ligand as there are no strongly upfield shifted CH_2 groups. A search of the histidine region of the NMR spectrum revealed only one histidine in its conventional position in all three oxidation states. The four remaining histidines are therefore iron ligands, and in what follows it will be assumed that the four haems are bound to eight of the nine histidines of the *vulgaris* sequence. The sequences of the five proteins in Table 1 strongly confirm this conclusion.

Details of NMR spectra

Using resolution enhancement techniques¹¹ (compare Fig. 3) the NMR resonance positions of at least one hundred resonances can be followed in each of the three oxidation states. In the fully oxidised state the temperature dependences of the resonances have been studied from 25° C to 55° C. This allows those resonances which are affected by the paramagnetic haem groups (temperature dependent) to be separated from those which are in their normal diamagnetic positions or are shifted from these positions by ring current effects (both temperature independent)⁷. The ring current shifts can then be related to known shifts observed in model compounds using the usual geometric relationships (Fig. 4). The paramagnetic shifted resonances can have been affected either by contact and pseudo-contact shift (this is the case for all groups linked by bonds to the haem iron) or by pseudo-contact shift alone (this is the case for all protein resonances except the groups bound directly to the iron).

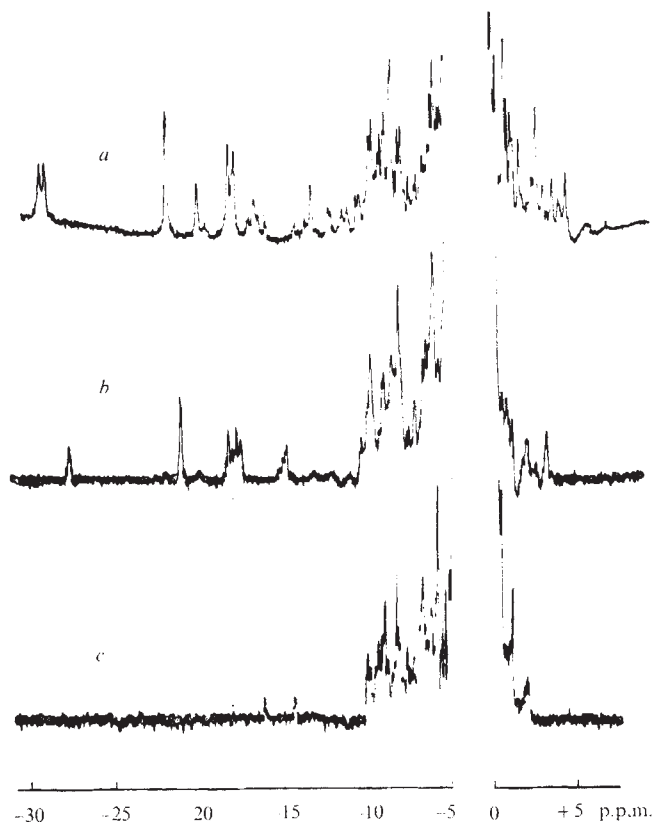


Fig. 2 NMR spectra (measured at 270 MHz) of *D. vulgaris* cytochrome c_3 in the various oxidation-reduction states observed during reduction with sodium dithionite. *a*, fully-oxidised species; *b*, half-reduced species; *c*, fully-reduced species. (Note the vertical scale in *b* and *c* is twice that in *a*.) The titration was carried out with 5 mM protein at 25° C. Dioxan was used as an internal standard but all peaks are referred to TSS.

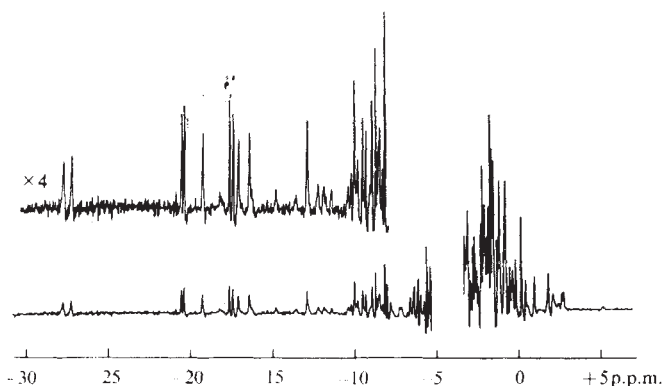


Fig. 3 Resolution enhanced spectrum of oxidised cytochrome c_3 at 50°C.

The pseudo-contact shifts contain much geometric information, which can be used in studies of structure^{4,10,12,13}. The greatest problem in using all the shift data, which undoubtedly permit a complete structure determination for this protein, is that of the assignment of the resonances to individual groups. Although we are not yet in a position to carry out such a detailed analysis we can give a qualitative interpretation of our data. We treat each of the three oxidation states in turn.

In the spectrum of fully oxidised cytochrome c_3 there are resonances from +15 to -30 p.p.m. as in cytochrome c , but the resonances are spread over the spectrum in a very different way from those in the cytochrome c spectrum⁷ despite the similarity in EPR g values⁸. The most obvious features are shown in Figs 2 and 3. Apart from the fact that there are the expected number of downfield, contact-shifted, haem-ring methyl group resonances (12 to 16 can be seen), there are very many other resonances with large shifts to both high- and low-field. Taking the high-field region first, resonances in this region which show a temperature-dependent shift must either lie in the neighbourhood of and outside the pseudo-contact 'cone' of the haem iron atoms (Fig. 4) or they must experience an appreciable contact shift. From models and studies on other haem proteins, three types of group on the porphyrin are known to give temperature dependent peaks shifted to high field⁷. They are the single proton haem methenes, some of the protons of the propionic acid side chains and at least one of the two methyl groups per haem in the groupings $\text{CH}_3\text{CH}(\text{haem})\text{S-protein}$, that is the methyl groups of the thioether links. As each of the four iron atoms is bound to two histidines, no other paramagnetically shifted methyl resonances of iron ligands are expected beyond +1.0 p.p.m. Furthermore, in the spectra of all the haem proteins so far examined, no paramagnetically shifted methyl group resonances of non-haem linked residues have been seen at fields higher than +1 p.p.m. In cytochrome c , excluding the resonance of the methionine methyl group which is known to be absent in the cytochrome c_3 proteins, the most strongly high-field shifted methyl resonances (due to a thioether methyl group) is seen at +2.2 p.p.m. (ref. 12). By contrast, cytochrome c_3 has some six methyl resonances in the region from +2 to +5 p.p.m. This large number of methyl groups shifted to high field leads immediately to the suggestion that some of the haems are placed end to end producing a region between them of large upfield pseudo-contact shift (see Figs 4, 5). In this region of the spectrum there are also a number of unidentified resonances of one or two protons intensity. Two single-proton peaks are very strongly upfield shifted (+15 p.p.m.) and probably arise from two methene protons.

In the low-field region, apart from the 12-16 contact shifted haem methyl group resonances, there are at least 10

resonances of one proton strength beyond -10 p.p.m. It is likely that these resonances are some of the C_2 and C_4CH groups of histidines which should be strongly downfield shifted by a pseudo-contact mechanism as they are inside the cone (Fig. 4).

The spectrum around -7 p.p.m. where the aromatic protons are generally observed is quite different from that expected, indicating that all or at least most of the five aromatic rings have their resonances displaced by pseudo-contact interactions. Similarly the methyl group resonances, which in a protein normally have strong maxima in the region of -1 to -3 p.p.m. are spread grossly over the whole range from -5 to -5 p.p.m. This is hardly surprising as each of the four haems will interact with some different part of the protein.

The region of the spectrum from -8 to -10 p.p.m. shows only one histidine resonance in roughly the correct position for a possible not bound to iron (see also ref. 10) but it also contains a large number of single proton peaks. We return to these resonances later for they must be very close to the haem group, as is shown by their broadening in intermediate states of oxidation-reduction. It is in this region of the spectrum that $>\text{NH}$ resonances are normally observed.

The resonance peaks of the half-oxidised species are readily separated from the fully reduced and fully oxidised

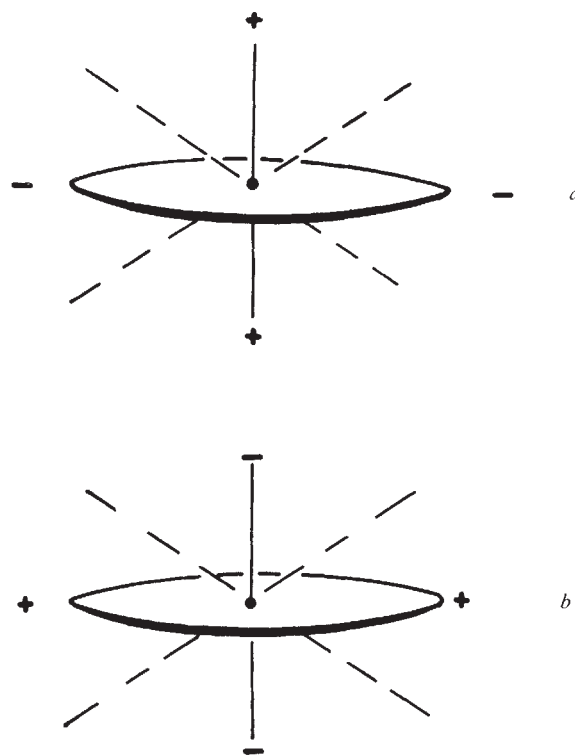


Fig. 4 Schematic representation of the regions of space (cones) which give rise to shifts of NMR resonances. *a*, The case for a ring-current shift which has axial symmetry. Protons lying within the cone experience an upfield shift (+). *b*, Case for axial pseudo-contact shift with the signs appropriate for low spin Fe(III) , a proton within the cone experiencing a downfield pseudo-contact shift. As the pseudo-contact term exceeds the ring-current shift by a factor of three, shifts in Fe(III) porphyrin complexes are in the opposed sense to those in diamagnetic Fe(II) porphyrin complexes. In a protein the field around Fe(II) is rhombic, though largely dominated by an axial term, so that care must be taken in the detailed interpretation of pseudo-contact shifts. The cones are derived from a consideration of the angle dependence of shift ($3 \cos^2\theta - 1$), where θ is the angle between r_i (the vector between the haem iron and the observed proton) and the g_z -axis.

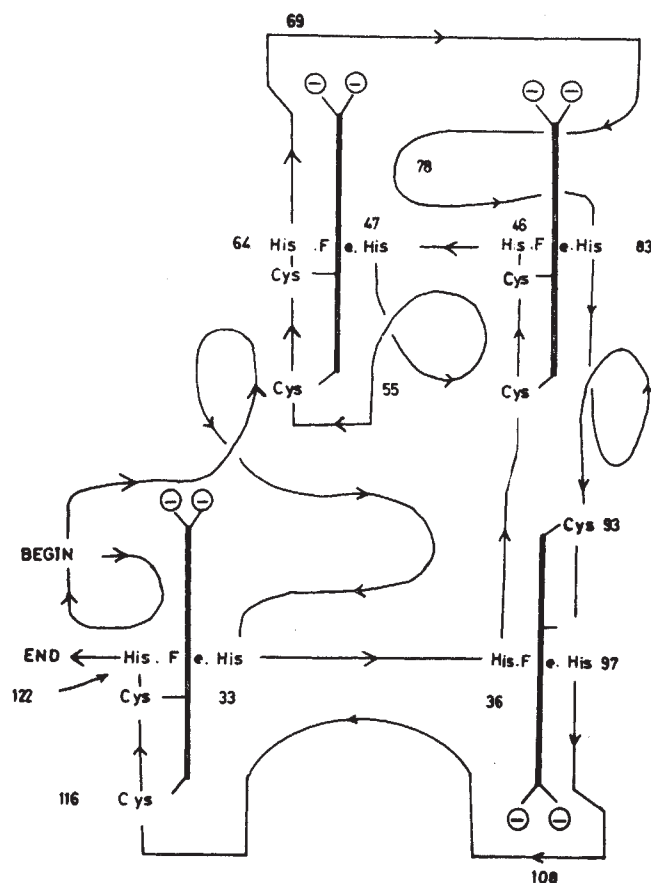


Fig. 5 Proposed outline structure of cytochrome c_3 from *Desulfovibrio vulgaris*.

species (Fig. 2). At very low field, beyond the normal aromatic proton region there are now some six to eight contact-shifted methyl resonances indicating that two haems remain oxidised. There are however only two methyl resonances at very high field, that is above +1.0 p.p.m., although there are also some broad and unidentified one and two proton peaks shifted into this region of the spectrum of oxidised cytochrome c , two paramagnetic haem groups replacing the one in cytochrome c , showing that the cooperativity required to explain the shifts in the fully oxidised species has disappeared. If two haems in the different oxidation states Fe(II), diamagnetic, and Fe(III), paramagnetic, are packed end to end then the shifts due to one are opposed to the shifts of the other (Fig. 4). The shifts are now anti-cooperative. We emphasise that the downfield contact-shifted methyl resonances move upfield going from fully oxidised to half reduced.

The aromatic protons of the half-oxidised spectrum are again not recognisable in their usual positions but the aliphatic region is more closely that of a conventional haem protein. A single histidine resonance lies in the expected part of the spectrum. The region from -8 to -10 p.p.m. has no structure but as in the oxidised species a rough estimate of the number of >NH protons which do not exchange in D_2O is five to ten.

The fully reduced spectrum has several unusual features. In the upfield region there are more than ten single resonances close to or above 0 p.p.m. We assign many of these single resonances to ring-current shifted protons of histidine bound to iron. They are shifted upfield by the ring current of the haem (Fig. 4). It is known that the α -protons of pyridine bound to a diamagnetic haem are shifted into this region¹⁴. There is only one possible histidine proton at -8

p.p.m. and thus as in the oxidised and intermediate forms we can state that eight of the nine histidines are bound to the haem. There are no methyl resonances in this upfield region. This shows yet again that unlike cytochrome c there are no methionines bound to the central iron. Furthermore, no methyl groups sit centrally inside the cone of the haem rings and close to the haem.

The methyl region, around -2 p.p.m. is now rather similar to that of other proteins. (McDonald and Phillips¹⁰ point out that in fact the methyl region is displaced to low field to some extent.) In the region between -6 and -10 p.p.m. there is a large number of sharp single proton resonances. It is hard to assess the number accurately but it exceeds 10. These are undoubtedly some of the ring methene bridge $\geq CH$ groups. They are downfield shifted by the ring current (Fig. 4). Again resonances from aromatic protons cannot be recognised in the usual positions. This is expected of course if the aromatic groups are close to the haem so that their resonances experience ring-current shifts. In this region, there is also a number of protons, say five to ten, assigned to nonexchanging >NH from peptide bonds. This region of the spectrum (in D_2O) is not altered after a cycle of oxidation and reduction or after cycling from 25° C to 55° C and holding at the high temperature for some hours, or even after several weeks at 0° C. Some of the >NH protons are very resistant to exchange.

This brings us to the most remarkable feature of the reduced spectrum. At very low field, -14.5 and -16.3 p.p.m., there are two single proton resonances. As this is a diamagnetic system such resonances must originate from >NH groups. It is not possible to conceive of a ring-current shift of minimally 8 p.p.m. downfield of the most downfield $\geq CH$ resonance. It is known that even the effect on ortho protons of haem-bound pyridine is only about 8 p.p.m. upfield¹⁴, and the chemical shift of imidazole protons of iron-haem ligands is about 0 p.p.m. As the maximum upfield shift is twice the maximum downfield shift, from the angle dependence of shift, $(3 \cos^2\theta - 1)$, (Fig. 4), we can eliminate the possibility of downfield shifts of greater than 5 p.p.m. We therefore assign the two resonances to >NH groups of histidine which are known to occur at such a low field in H_2O (ref. 15). Usually such resonances exchange rapidly in D_2O . These two histidines must be in a region of the protein very well protected from water. Since their resonances disappear from this part of the spectrum on oxidation. (Figs 1 and 2), they must be bound to iron.

Introduction of very small amounts of either oxidising or reducing agents to the fully reduced or fully oxidised systems respectively causes a profound change in the line widths of some resonances. In fact all the sharp resonances due to haem protons, for example methene $\geq CH$, broadened on altering the redox state of either the fully reduced or fully oxidised protein by less than 10%. These resonances include those assigned to the bound histidines as well as the protons of the haem itself. All the haems seem to be affected. Further oxidation and reduction towards the intermediate form broadens all the resonances with the result that the half-oxidised state gives a relatively poor spectrum in which it is hard to find such resonances as those of the methene bridge protons or those of the histidines. But during oxidation or reduction, well separated resonances of each of the three species titrate without shifting showing that equilibrium between oxidised and reduced species is not fast (on the NMR time scale) for otherwise intermediate resonance positions would be seen.

The broadening of resonances of haem proteins must arise however from relatively fast chemical exchange as the electron relaxation rate of low-spin haem is known to be fast. There are two electron exchange rates to consider: (1) internal exchange between haems of the same protein molecule and (2) exchange between different protein

molecules. A full study of the reaction rates with temperature and concentration will give much knowledge of the electron transfer process in proteins but it is clear that the electron exchange is of intermediate rate rather than very fast or very slow.

Structural features

The sequence and the NMR data on *Desulfovibrio vulgaris* cytochrome c_3 can now be put together. The short lengths of sequence between haem-bound histidines at residues 46–47 and 33–36 force the planes of the haems to be roughly parallel pairs. Each haem is bound to two histidines and to two thioether links. At least some of the haems must also be close together and stacked end to end as the large cooperative upfield shifts of the methyls of the thioether bridges in the fully oxidised state indicates. The anticooperativity of ring-current and pseudo-contact interactions of the haems provided by the arrangement in Fig. 5 also fits observations from the data on the half-oxidised and fully-reduced states. The histidine 80 which is not haem-bound and is found in a normal position in the NMR spectrum is only found in *Desulfovibrio vulgaris*¹⁰. The aromatic groups must now be placed reasonably close to the haem centres for they do not appear as normal aromatic resonances in the NMR spectra of any of the three forms we have studied. It is also known that in haem proteins it is usual for the two carboxylate residues of the haem to be neutralised by basic groups in their vicinity. In Fig. 5 we have placed invariant basic residues close to the carboxylate groups. McDonald and Phillips¹⁰ point out that the NMR spectra indicate that the lysines are on the outside of the molecule.

The model agrees with the observation on *Chloropseudomonas ethylica* that many (26) amino acids from 55 to 90 and histidine 47 which binds to the haem in the top left hand corner of Fig. 5 are missing. In the three-haem cytochromes this haem must be absent. We have built a working three-dimensional model of the structure to prove that it fills space in a satisfactory way. The distance between haems is about 10 Å, in agreement with assessments of this distance based on one interpretation of the optical spectroscopy^{9,10}.

Further details of the structure can be unravelled by a thorough assignment of the resonances, and a calculation of the resonance shifts together with model building. Further NMR probes can be introduced as becomes necessary.

The most interesting feature of the protein is that despite the fact that the haems are close together (about 10 Å apart) and are separated by only a single polypeptide

chain (Fig. 5) there is relatively slow electron exchange between them. Thus such a protein is not a simple electron conductor but is more likely a storage unit, a capacitor, for electrons. It cannot be the case that normal amino acid residues can give rise to a path for rapid electron exchange. In a model experiment we have shown that collision between low-spin Fe(II) and Fe(III) haems provides fast exchange¹⁷. Thus the haems in cytochrome c_3 (as in other cytochromes) are not able to come into close contact through intermolecular collisions. The possibility remains that these proteins are devices for multielectron reactions in which electrons are released one at a time but in rapid succession.

C. C. McDonald and W. D. Phillips have measured¹⁰ independently the NMR spectrum of cytochrome c_3 . Their data and deductions are very similar to ours. We wish to acknowledge their friendly cooperation. R.J.P.W. is a member of the Oxford enzyme group. C.F.G. acknowledges the receipt of a NATO grant. We thank Dr R. P. Ambler for permission to reproduce the sequences given in Table 1.

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LETTERS TO NATURE

PHYSICAL SCIENCES

Shell flashes in planetary nuclei

THE central stars of planetary nebulae may be the ancestors of some of the white dwarfs^{1–3}, and the magnetic white dwarfs in particular may have originated in this way^{4,5}. There seem to be no mechanisms in the white dwarfs themselves capable

of producing fields of the strengths observed, and the magnetic field must therefore be a fossil remnant of an earlier phase of evolution. The hypothesis that a high internal field is exposed by the ejection of the nebular shell has an appealing simplicity.

A satisfactory explanation for the postulated high internal fields in the planetary nuclei has not yet been suggested. Conservation of magnetic flux from the main sequence phase of evolution is adequate only if the main sequence progenitors of the planetaries have average field intensities $B \sim 10^3$

gauss. Such fields do exist in the magnetic A stars, but they seem to be too massive to be the ancestors of the planetaries.

We point out here that a natural mechanism for the production of strong magnetic fields may in fact be present in at least some planetary nuclei. A prominent feature of the evolution of such stars is the occurrence of one or more thermal runaways, 'flashes', in an unstable shell source which produces nuclear energy⁶. Near the peak of a flash a transient convection zone is invariably developed⁷⁻¹¹, and if the star has even a modest amount of differential rotation, the generation of magnetic field in a stellar dynamo can occur. This hypothesis was suggested to us by the recent paper of Ruderman and Sutherland¹², in which it is argued that the transient convection accompanying the explosive burning of carbon in carbon-detonation supernovae, will tangle up any initial 'seed' magnetic field and perhaps cause amplification to the equipartition field strength.

In order to investigate the plausibility of dynamo field generation in the planetaries, it is necessary to have estimates of the rate of differential rotation and of the properties of the transient convection zones in these stars. We are unaware of any determinations of the rotation of planetary nuclei. One of the magnetic white dwarfs is, however, known to rotate with a period, P , of 1.33 d (ref. 13). If this star has indeed evolved from a planetary nucleus, conservation of angular momentum implies a period $P \sim 10^2$ d for the ancestor of the white dwarf. This value seems conservative, and we therefore adopt it for our calculations.

As even less is known about the differential rotation of planetary nuclei, we adopt $\Delta P/P \sim 0.1$, the value appropriate to the sun, where ΔP is the magnitude of the difference in rotation period between the pole and the equator.

Because the precise details of the evolutionary history of the planetaries are uncertain, the exact structure of such stars immediately before the expulsion of the nebular shell is unknown⁶. As a result, a considerable variety of models have been suggested for these objects. Thus, Rose and Smith⁷ and Paczynski⁸ consider instabilities in the He-shell of double-shell-source models; Wood and Faulkner⁹ consider the shell-flash in pure helium stars; Joss *et al.*¹⁰ investigate models in which either He- or C-shell flashes can occur; and Kutter and Savedoff¹¹ consider shell flashes in pure carbon stars. Unfortunately, except in the latter paper, only very schematic information is given about the structure of the convection zones, and we therefore rely on rather crude estimates for the convective velocities.

The following results are typical of these models. For models that exhibit a He-shell flash⁷⁻¹⁰: the stellar mass is $M \sim 0.8 M_\odot$; the mass of the helium envelope—which is almost entirely convective at the peak of the flash—is $\sim 2 \times 10^{-3} M_\odot$ (the convection zone may, however, cover a substantial fraction ($\sim 1/2$) of the stellar radius; the density at the He-burning shell ρ is $\sim 2 \times 10^3$ g cm⁻³; the convective velocity v is $\sim 3 \times 10^5$ cm s⁻¹; and the duration of the transient convection is ~ 10 – 100 yr. For models that exhibit a C-shell flash^{10,11}: the stellar mass is $\sim 1.1 M_\odot$; convection covers $\sim 1/3$ of the stellar mass; the density at the C-burning shell ρ is $\sim 10^5$ g cm⁻³, the convective velocity v is $\sim 10^5$ cm s⁻¹, and the duration of the transient convection is $\sim 2 \times 10^2$ yr. In either case the stellar radii R are $\sim 0.1 R_\odot$ (as they must before planetary nuclei) and the equipartition magnetic field strengths B_{eq} , are $\sim (8\pi \cdot (1/2) \rho v^2)^{1/2} \sim 10^7$ – 10^8 gauss. This is more than adequate to account for the magnetic fields of $\sim 10^7$ gauss detected in the magnetic white dwarfs (which have radii of about $\sim 10^{-2} R_\odot$), provided that the transient convection lasts long enough for the dynamo to generate such fields.

In the conventional picture of a dynamo¹⁴, an initial poloidal 'seed' field of intensity B_0 is distorted by the differential rotation to produce a toroidal field component. This is a continuing process, with the toroidal field doubling in a time $\tau \sim P^2/\Delta P$. At the same time a new poloidal field is generated by the action of the cyclonic convection, which lifts and twists

the toroidal field lines¹⁴. Growth of the field is ultimately halted when the magnetic intensity approaches the equipartition field strength, B_{eq} ; not only is the convection then too weak to deform the field lines, but also the stronger field suppresses the convective motions.

That this schematic picture is qualitatively correct can readily be seen by applying it to the Sun. In the solar convection zone, $B_{eq} \sim 4 \times 10^3$ gauss, which is the order of magnitude of the fields seen in sunspots, where magnetic field lines from the interior protrude through the solar photosphere. Furthermore, the doubling time is $\tau \sim 1$ yr, and the time required for an initial 'seed' field of intensity $B_0 \sim 1$ gauss (the general solar field) to reach this limiting intensity is $t \sim \tau \log_{10}(B_{eq}/B_0)/\log_{10}2 \sim 10$ yr, which is almost exactly half the sunspot cycle.

For the planetary nuclei, our previous estimates of the differential rotation indicate a doubling time $\tau \sim 3$ yr. If the initial 'seed' field is again taken to be $B_0 \sim 1$ gauss (it would be $\sim 10^2$ gauss if flux conservation from the main sequence were assumed), the time required for the field to reach its equipartition strength of $\sim 10^7$ – 10^8 gauss is $t \sim \tau \log_{10}(B_{eq}/B_0)/\log_{10}2 \sim 70$ – 80 yr. This is comparable with the duration of the transient convection zone, and it therefore seems entirely possible that fields of the intensity ($\sim 10^6$ gauss) required in order to explain the magnetic white dwarfs can be generated in at least some planetaries.

It is tempting to suggest that the production of a magnetic field necessarily accompanies the ejection of a planetary nebula or even, perhaps, that nebular ejection is caused by the production of strong magnetic fields. This is certainly premature at present, although if the latter conjecture were true, all planetary nuclei should have strong magnetic fields, and it should be possible to verify this by observation.

If dynamo generation of strong magnetic fields actually occurs during shell flashes in planetary nuclei, as we have suggested, a prime candidate for the observational test of this hypothesis would seem to be the remarkable star FG Sge. This star is known to have ejected a planetary nebula $\sim 6,000$ yr ago, and it seems to be undergoing an active shell flash at the present (refs 8, 15, and G. E. Langer, *et al.*, unpublished information). Most remarkable, however, is the recent discovery (G. E. Langer, *et al.*, unpublished information) that the surface layers are currently being enriched in s-process nuclei, which are thought to be produced in the He-shell flash. Along with these nuclei strong magnetic fields would also be expected to be transported to the surface if our hypothesis is correct. Measurements of the magnetic field of this star thus seem to present a crucial test of this idea.

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H. M. VAN HORN*
C. J. HANSEN

Joint Institute for Laboratory Astrophysics,
University of Colorado and National Bureau of Standards,
Boulder,
Colorado 80302

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*On leave from Department of Physics and Astronomy, University of Rochester, USA.

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Gravity-induced electric polarisation near the Schwarzschild limit

It has long been recognised that besides carrying the fundamental unit of charge an electron is also endowed with inertia. Only recently has attention been paid to the possibility that phenomena may exist which depend on the gravitational mass¹. Here we point out that strong gravitational fields, such as those encountered in collapsed stellar objects, may produce observable electromagnetic effects arising directly from the gravitational mass of electric charges.

Some sixty years ago Tolman and Stewart² described an experiment in which the inertia of electrons is revealed. This involved a conductor, such as a circular horizontal wire, which oscillates about a vertical through its centre. Because of their inertia the free electrons will move relative to the crystal structure of the wire and so an oscillating current will be generated. To a first approximation, if $\mathbf{a}$ is the acceleration of the wire at any instant, then as far as the free electrons are concerned an effective electric field $\mathbf{E}$ given by

$$\mathbf{E} = \left(\frac{m}{e} \right) \mathbf{a} \quad (1)$$

is present in the reference frame of the wire (m and e are the electronic mass and charge, respectively). In principle a method for obtaining the specific charge e/m was made available.

From the point of view of relativity theory, we may regard the acceleration of the wire as equivalent to the action of a gravitational field (of oscillatory character in the above case), in accordance with Einstein's principle of equivalence. In fact whenever a conductor is held in a gravitational field $\mathbf{g}$ we may expect an effective electric field

$$\mathbf{E} = - \left(\frac{m}{e} \right) \mathbf{g} \quad (2)$$

to be manifested to the free electrons. In this case m is the electron's gravitational mass, indistinguishable from the inertial mass in the light of the equivalence principle.

If this is the effect of gravitation on free electrons (admittedly a very small one in the gravitational field of the Earth) then a corresponding phenomenon must influence bound charges in a dielectric. We may think of this as a gravitational polarisation

$$\mathbf{P} = \chi \mathbf{E} = - \chi \left(\frac{m}{e} \right) \mathbf{g} \quad (3)$$

where χ is the gravitational susceptibility of the dielectric. If indeed $\mathbf{P}$ exists then, as in ordinary electric polarisation, an equivalent volume distribution of charge $-\text{div. } \mathbf{P}$ and surface distribution $\mathbf{P} \cdot \mathbf{n}$ would result in accordance with conventional analysis.

For the gravitational field of the Earth such a polarisation no doubt produces extremely small consequences. But in a collapsed star lying near to its Schwarzschild limit the local value of $\mathbf{g}$ may be large enough to produce observable effects, especially in conjunction with a rapid rotation of the star³. Thus if we apply the usual Schwarzschild metric to a condensed object then the local value of the gravitational intensity on the surface $R = R_s$ is given by

$$g = (GM / R_s^2) (1 - [2GM / (c^2 R_s)])^{-1/2} \quad (4)$$

M being the total mass and G the Newtonian gravitational

constant. Here g is the proper acceleration (invariant) of a freely falling test particle relative to the fixed surface, and is equal to the magnitude of the first curvature vector of the world line of an atom fixed under stress at $R = R_s$. Equivalently g is the acceleration (in the opposite direction) of the fixed atom relative to a freely falling geodesic frame instantaneously at rest at $R = R_s$. Clearly if $R_s = R^*$, where $R^* = 2GM/c^2$ (the Schwarzschild gravitational radius) g may become extremely large, with corresponding intensification of the effects we have described on free and bound electric charges. Even in a neutron star (the limiting consequence of these effects) there will be such charges near the surface.

We now consider in particular what happens to a 'standard clock' S located in such intense gravitational fields. If the standard clock is an atomic emission line then we cannot expect this to be at the standard laboratory frequency in such circumstances. There must necessarily be an effect related to the electric Stark phenomenon. For example although the usual redshift formula will apply to the emitted radiation as received by a remote observer O , it is not sufficiently realised that this formula gives only the ratio v_s / v_o for the frequencies emitted and observed in terms of the change in the gravitational field between S and O . It tells us nothing about the emitted frequency absolutely. The emitted frequency will in fact be standard only for an atom in free fall, or when the gravitational field is negligible. It is usually assumed in the case of the Sun, for example, that these conditions prevail. But clearly the assumption is invalid when we consider an atom fixed on the surface of a highly condensed object.

W. DAVIDSON
H. J. EFINGER

Department of Mathematics,
University of Otago,
Dunedin, New Zealand

Received January 23, 1974.

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Meteorological features affecting large aperture synthesis radio telescopes

VARIATIONS of atmospheric radio refractive index can cause phase differences between signals received at spaced aerials and limit the resolution of large radio telescopes using the aperture synthesis technique¹. Hinder² found that anomalous phase differences recorded at the One-Mile telescope at Cambridge³ were caused by thermal updraughts and fair weather cumulus; the mean size of the irregularities was 700 m. Earlier calculations⁴ had shown that the gradients of refractive index across fronts were likely to be important but it was believed that corrections using ground-based measurements of pressure, temperature, and humidity could be applied to reduce errors from such large scale (≈ 500 km) weather systems⁵.

The first few months of observing with the 5-km telescope at the Mullard Radio Astronomy Observatory, University of Cambridge, showed that there were frequent significant effects from intermediate systems, with scales of 10 to 100 km or more. An investigation was undertaken to relate these periods of anomalous phase changes—'events'—to meteorological phenomena and the results are summarised here.

Telescope operational periods (excluding those spent observing complex emitting regions) event times to within 1 h and event intensities, for part of May, June to September, and

Table 1 Event distribution by month and time of day

Time GMT	May*		June		July		August		September		December		May - September		
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(c)
00 – 06	111	242	198	675	128	535	109	607	64	483	35	580	610	2542	24.0
06 – 12	52	173	45	267	13	347	106.5	455	62	363	30.5	621	278.5	1605	17.3
12 – 18	50	134	42	485	174	548	142	733	83	419	117.5	740	491	2319	21.2
18 – 24	20	247	260	873	92	733	164.5	866	85	535	25	699	621.5	3254	19.1
Total	233	796	545	2300	407	2163	522	2661	294	1800	208	2640	2001	9720	20.6
(c)	29.3		23.7		18.8		19.6		16.3		7.9		20.6		
Nos of Events	9		18		13		19		10		10		69		
Intensity															
Max.	0.67		0.89		0.87		0.93		0.58		0.33		0.93		
Min.	0.32		0.24		0.14		0.19		0.17		0.10		0.14		
Mean.	0.44		0.50		0.47		0.46		0.34		0.23		0.45		
Medan	0.41		0.46		0.44		0.41		0.32		0.24		0.42		

(a) Total duration of events in tenths of an hour. (b) Period of operation of telescope in tenths of an hour. (c) % of operational time occupied by events.

* May data commenced on May 21.

December 1972 were considered. The intensity of an event is a normalised measure of the rate of increase in phase shift with aerial separation with first order geometrical differences removed. The observational programme was independent of weather conditions.

Table 1 gives the distribution of events by month and time of day. The data for December show that in winter, when the absolute water content of the atmosphere is at a minimum, events are likely to pose less of a problem, so the analysis was confined to the period May to September.

A classification of vertical variations of temperature, based on radiosonde reports for Hemsby (52° 41' N, 01° 41' E) and Crawley (51° 05' N, 0° 13' W), showed no relationship with occurrence of events; however, some events appeared to be associated with sharp vertical gradients of humidity marking the lower boundary of very dry mid-tropospheric layers. Such layers are associated with fronts⁶ and may persist long after the front becomes inactive; such an association implies a sloping interface and hence a much larger horizontal gradient of radio refractive index than might be expected with more extensive dry layers in anticyclones.

indeed a 12 h operational period from 1745 GMT on 8 September marked by continuous, sometimes heavy, rain throughout was free of events. Recent investigations into the structure of frontal systems^{7,8} have established the existence of rain bands of approximate scale 100 × 30 km, within which are 'clusters' of order 20 × 30 km marked by higher rainfall rates; these could explain the relationship between moderate and heavy rainfall and events. The prolonged rain of September 8 and 9, mentioned above, did not seem to conform to this model. On that occasion rapid and intense cyclonic development occurred off South-west England, the resulting depression passing near Cambridge. In the early stages of a development of such intensity, the precipitation structure would not be expected to have consisted of organised cluster bands. Events were also noted during periods of convective activity. This is not surprising in view of the large humidity contrast between the upward rising convective columns and the neighbouring drier environment, and typical variations⁹ in the depth of the convective boundary layer on distance scales of tens of km.

This investigation was limited by the lack of fine scale meteorological data, particularly upper air observations, from

Table 2 Precipitation at Cardington during operational periods at Cambridge (May to September only)

	Moderate or heavy rain	Thunder	Showers	Slight rain	Dry	Total
Event	3.9	1.0	18.6	12.2	164.4	200.1
No event	6.1	2.0	31.9	48.9	683.0	771.9
% of time occupied by events	39.0	33.3	36.8	20.0	19.4	20.6

All values are given in hours and tenths.

Considerations of 12-h changes in air mass characteristics at Hemsby and Crawley, and pressure variations recorded by the microbarogram at Kew Observatory, were inconclusive; in both cases only the few large changes showed any possible association with events. No additional relationships were found from a study of more detailed vertical soundings in the lowest 1,200 m, carried out four times daily at Cardington (52° 06' N 00° 25' W).

When hourly weather reports from Cardington were scrutinised, some events were found to coincide with the onset or cessation of rain. Table 2 gives the total times in hours and tenths when various precipitation types and intensities occurred in operational periods with and without events. If slight rain is disregarded, the relationship between events and other precipitation types is significant at the 0.1% level. No event in September coincided with precipitation (at Cardington);

the vicinity of the telescope itself; a detailed synoptic study would require surface and upper air observations at the site. But it has indicated three systems which may have caused many of the phase differences observed: rain 'clusters'—usually associated with fronts, areas of showery activity and occasional very dry mid-tropospheric layers. These systems are all associated with humidity or liquid water content variations in the vertical and the horizontal, which are not limited to the lowest layers of the atmosphere. Therefore the likely frequencies of such systems must be taken into account when considering sites for new radio telescopes.

R. N. HARDY

*Meteorological Office,
London Road,
Bracknell, Berkshire RE12 2SZ, UK*

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Neutral oxygen in comets

MEASUREMENTS of the strength of permitted spectral lines from neutral oxygen in comets should provide both a better understanding of gas dynamics in comets and an assessment of whether the production ratio of oxygen to hydrogen in comet atmospheres is close to a half. If this ratio is confirmed, it would provide strong support for the growing evidence¹ that the 'parent' molecules which make up the solid comet nucleus are dominantly H₂O.

A better understanding of the role of H₂O in comets can be obtained from the study of both atomic oxygen and hydrogen. Most observations of atomic oxygen in comets seem to be of the forbidden lines at 6,300 Å and 6,363 Å. These lines presumably occur only in cases in which an oxygen atom was left in the ¹D₂ level at the time of dissociation, and they are thus related to the production rate of atomic oxygen rather than to the oxygen density (ref. 2). Measurement of permitted oxygen lines which result from photoexcitation by solar photons would provide a direct probe of neutral oxygen density.

Photoexcitation of neutral oxygen from the 2p⁴ ³P₂ ground state to the 3s ³S₀ level gives rise to three resonance-fluorescence lines at 1,302 Å, 1,305 Å and 1,306 Å. These lines have been observed in the upper atmospheres of both Venus and Mars^{3,4} and in Comet Kohoutek⁵. The fact that the same OI lines appear in emission in the solar chromosphere can considerably enhance the fluorescence. In a cometary atmosphere, however, the neutral oxygen may not be excited by these lines if the radial velocity of the comet with respect to the Sun is large enough to Doppler shift the wavelength of the exciting lines. In this case the excitation would be expected to be by the solar continuum, which is of a much lower intensity than the chromospheric OI lines. There is, however, another method of exciting OI in a comet. This is possible because of the almost exact coincidence of the oxygen lines at 1,025.77 Å and the Lyman-β hydrogen line at 1,025.72 Å, which appears in emission in the solar chromosphere (see also ref. 6). Lyman-β has a width of approximately 0.4 Å in the Sun, and therefore the radial velocity of the comet with respect to the sun will not be enough to prevent the occurrence of fluorescence.

The neutral oxygen atoms in the 3p⁴ ³P₂ ground state are excited to the 3d ³D_{3,2,1} states by Lyman-β photons. The excited OI atoms can return to the ground state either by direct transitions, or through transitions to the 3p ³P and 3s ³S⁰ states, which will give rise to lines at 11,287 Å, 8,446.5 Å and the three lines near 1,304 Å. There is thus the interesting possibility of detecting the presence of neutral atomic oxygen in comets, from allowed transitions in the near infrared spectrum.

The concentration of neutral oxygen in the atmosphere of a comet will depend on the rate of production, the rate of escape from the nuclear region and the rate of removal by ionisation. The rate of production of oxygen can be deduced if it is assumed that the hydrogen cloud around a comet is the result of photodissociation of water vapour, in which case the rate of production of oxygen should be half of the rate of production of hydrogen.

The rate of escape of oxygen from the nuclear region is difficult to estimate accurately, because there is evidence that collisions in the region near the nucleus may be rather frequent⁷, resulting in an equipartition of energy between the component particles. In this case the outflow velocity of oxygen atoms into the coma would be a quarter of the hydrogen velocity. For oxygen atoms released by photodissociation of OH at a distance from the nucleus, conservation of momentum imparts a velocity to the oxygen atoms of one sixteenth that of hydrogen.

The ionisation of hydrogen in comets is predominantly a result of charge exchange by protons in the solar wind. This mechanism is about 10 times more important than photoionisation⁸. The lifetime against photoionisation of oxygen by solar photons (found from the cross sections in ref. 9) is very close to that for hydrogen. The cross section to ionise oxygen by charge exchange with solar wind protons is, however, two to three times smaller than the hydrogen cross section¹⁰, so the total lifetime of neutral oxygen atoms is still determined by the lifetime against charge exchange. The smaller charge-exchange cross section of oxygen allows most of the oxygen atoms from a comet 1 AU from the Sun to remain neutral out to at least 10⁶ km from the nucleus, in spite of their lower velocities. At a distance of 10⁶ km from the nucleus, the oxygen density is about two to five times that of hydrogen, for hydrogen to oxygen velocity ratios of 4 and 16 respectively, assuming that the hydrogen and oxygen atoms both come from H₂O.

From the published transition probabilities¹¹ for O and H the photon flux of the 8,446.5 Å OI line from a comet can be estimated in terms of the 6,563 Å Hα line, because both are nearly proportional to the Lyman-β flux from the Sun. The calculated photon flux for the 8,446.5 Å OI line is 0.8 to 2.0 of the Hα flux, when the oxygen density is two and five times the hydrogen density respectively. This assumes that the comet is optically thin to the solar Lyman-β photons, but as both the oxygen and hydrogen clouds in the comet are expected to be considerably larger than the radius at which Lyman-β becomes optically thick (about 10⁵ km) (ref. 12), it seems justifiable to assume a small optical depth.

The observation of Hα in Comet Kohoutek, using a Fabry-Perot interferometer¹³, suggests that it might be possible to observe the OI line at 8,446.5 Å using an instrument with a high enough resolution to discriminate against the continuous background spectrum. Such observations, together with more observations of the far ultraviolet spectra of hydrogen and oxygen, the OH emission at 3080 Å, and the forbidden oxygen lines at 6,300 Å and 6,363 Å, may provide sufficient information on the physical and dynamical processes occurring in comets to allow a critical evaluation of the importance of H₂O in the dirty-ice model.

P. L. BYARD
G. H. NEWSOM

Department of Astronomy,
The Ohio State University,
Columbus, Ohio 43210

Received February 18, 1974.

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PAN over the Atlantic and the smell of clean linen

OZONE, at concentrations in excess of 0.1 parts per million (p.p.m.), has been observed in the summertime air over southern England¹. The most probable source is photochemical reaction among air pollution products. We therefore sought the presence of another characteristic product of urban atmospheric photochemistry², peroxy acetyl nitrate (PAN). Preliminary measurements (S. A. Penkett and F. J. Sandalls, personal communication) show that PAN is present in the air over southern England. During episodes of increased ozone and other atmospheric pollution indicators the concentration increased, but as with ozone there was always a background concentration of PAN even in clean air arriving from the Atlantic. The voyage of the German Research Vessel Meteor from Hamburg to Santo Domingo in October 1973 presented an opportunity to discover whether or not there is a natural background of PAN.

Analysis was by gas chromatography using an electron capture detector. Air samples were collected at the bows of the ship by drawing 5 ml of air into a glass syringe; the sample was then quickly injected into the gas chromatograph. The apparent concentration of PAN during the voyage is shown in Fig. 1. Concentrations of CCl_3F are also included because increases in the background level of this compound indicate the presence of air from urban industrial regions³. The term 'apparent' is used because it was discovered during the voyage that the unexpectedly high concentrations over the Atlantic were not present in the air, as such, but had formed by reaction on the glass surface of the sampling syringe. No PAN could be detected in air collected in polypropylene syringes, but the addition of a twist of glass wool to such syringes before the air was drawn into them restored the concentration to levels similar to those found with glass syringes. One-litre samples of fresh air were also drawn through plugs containing 1 g of glass or cotton wool, and these were found to be strong sources of PAN and of peroxy propionyl nitrate (PPN). In addition the wools possessed the characteristic bleach-like odour of linen after it has been dried in the open air. The PAN potentials of Fig. 1 are approximate and probably too low. No corrections were made for losses by adsorption or for the extent to which the detector was not coulometric.

The potential for formation of PAN was most marked on days of unobstructed sunshine (see Fig. 1). October 13 and 14, when the ship passed through a polluted air mass (which was indicated by the high concentrations of CCl_3F) were exceptions. PAN formation was greatest at approximately 1400 h and was between 3 and 10 times lower at

dawn and in the evening. Simultaneous measurements from the ship, of the aerial concentration of atmospheric hydrocarbons, indicated levels of between 0.1 and 2 parts per 10⁹ (p.p.b.) for the lower unsaturated hydrocarbons (R. A. Rasmussen, personal communication). The sea was apparently the most probable source. The concentration of

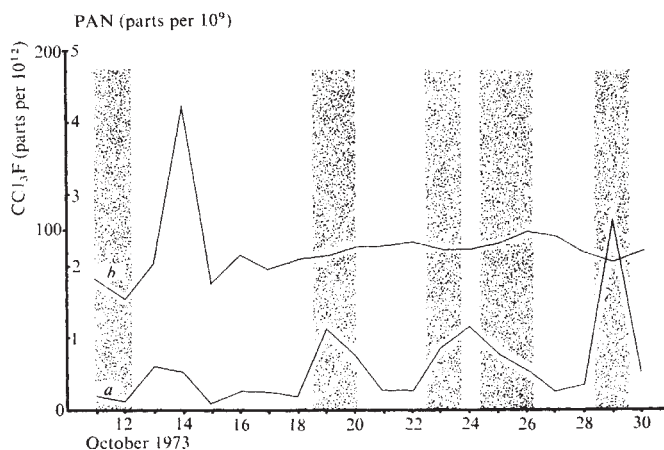


Fig. 1 PAN from the air over the Atlantic. Average daily concentrations of 'apparent' PAN $\times 10^{-9}$ (a) and $\text{CCl}_3\text{F} \times 10^{-12}$ (b) by volume. Shaded columns indicate days with less than 50% cloud cover.

the other ingredients necessary to form PAN by photochemical reaction were not measured. Ozone is ubiquitous and can be presumed to have been present in at least its background concentration of about 50 p.p.b.

These preliminary observations suggested that PAN and similar compounds can be formed in the clean air of remote maritime regions by heterogeneous reaction if a suitable surface is provided. They raise some interesting and important questions. What are the mid-Atlantic precursors? Is PAN formation in urban photochemical smog also by heterogeneous reaction? If so what is the surface on which it forms? Finally the quantities of PAN observed suggest the presence of a high background concentration of NO_x or of some related molecular species.

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JAMES E. LOVELOCK

Department of Applied Physical Sciences,
Reading University,
Whiteknights,
Reading RG6 2AL, UK

STUART A. PENKETT

Health Physics and Medical Division,
Atomic Energy Research Establishment,
Harwell, Berkshire, UK

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Vertical distribution of heat production in the Basement of the Eastern Alps

A LINEAR relationship between heat flow and heat production within plutonic bodies has been observed by a number of researchers^{1,2} and can be written:

$$q = q^* + DA(0) \quad (1)$$

where q is the surface heat flow, $A(0)$ is the surface heat production, and q^* and D are constants within a given geographic province.

Many heat production–depth models satisfy equation (1), but Lachenbruch^{2,3} suggests that an exponential model (equation (2)) is needed if equation (1) is to be preserved after differential erosion of the source layer.

Thus, the following equation is initially assumed to be valid for the area under discussion:

$$A(z) = A(0) \exp(-z/D) \quad 0 < z \leq z^* \quad (2)$$

Data are therefore presented in the form:

$$\log_e A(z) = \log_e A(0) - z/D$$

$$\text{or } z = -D \log_e A(z) + D \log_e A(0) \quad (3)$$

The distribution of heat production with depth in a granitic terrain is discussed here and the results of this direct measurement approach are compared with those already obtained from heat flow data elsewhere. The granitic rocks (the Zentralgneis) from the Pennine Basement of the Tauern Window in Austria were chosen because of the excellent exposure and because a vertical section of 4 km is available for sampling.

The Zentralgneis is a Variscan polyphase intrusive series of tonalites, granodiorites, and granites, which has undergone deformation, and subsequent amphibolite facies metamorphism during the Tertiary. A representative sample of the lithologies within each unit 1 km thick was obtained whenever possible. Particular care was taken to ensure that each unit was represented by a similar number of samples. Measurement of the amounts of uranium, thorium, and potassium was carried out using standard γ -ray spectrometry techniques and heat production was calculated from these data using the formula published by Birch *et al.*¹.

The depth of each sample (m) was calculated from a map published by Cliff *et al.* (ref. 4, page 234) on which the contours of the projected base of the overlying unit, the Peripheral Schieferhülle, are extrapolated over this area. Errors are difficult to estimate but are thought to be no greater than ± 250 m over the whole of the sampled area.

Heat production falls off markedly with increasing depth (Fig. 1); but the scatter of data points is such that it is not possible to distinguish an exponential model from, for example, a linear diminution of heat production with depth. The same problem of a high background noise on the fitted curve also led Lachenbruch and Bunker⁵ to an identical conclusion in the only other attempt to verify the exponential model by direct measurement.

In both cases, the difference in scale between the thermal equilibrium, which enables a heat flow value to be representative of large volumes of rock, and the petrological and geochemical equilibria, which are commonly confined to a sample the size of a hand specimen in such terrains appears unbridgable. But heat flow values tend to smooth out the small scale chemical inhomogeneities and, it is therefore possible that such a geophysical approach will add a new large scale dimension to the petrogenetic studies of composite intrusive series.

The second important result is that, although heat production does fall off with depth as predicted by Lachenbruch's interpretation of the heat flow data, it falls off considerably faster in the Zentralgneis than in any other province so far investigated.

Lachenbruch has explained the linear relationship described by equation (1) in terms of a steady state one-dimensional model, assuming the lateral gradients in heat production to be negligible compared with the vertical gradients. Consequently, the vertical section of 4 km in the Zentralgneis has been subdivided into four horizontal units 1 km thick and the

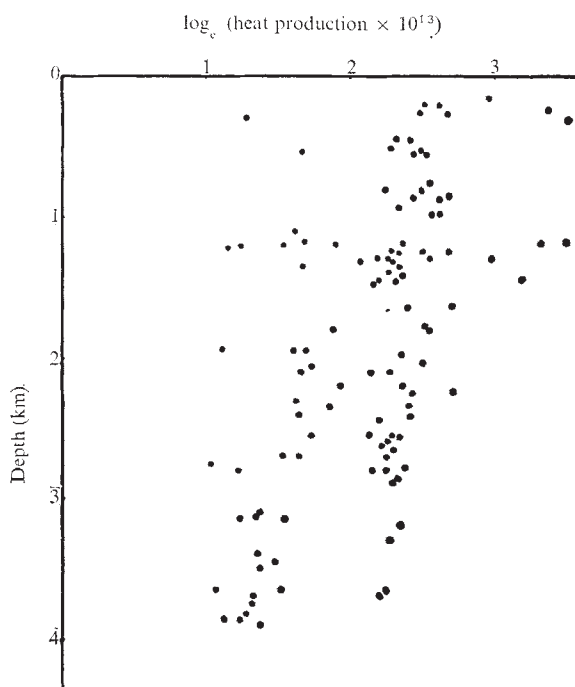


Fig. 1 Distribution of $\log_e$ (heat production $\times 10^{13}$) with depth (km) below the top of the basement complex in the Tauern Window. All samples were taken from the metaplutonic rocks of the Zentralgneis.

mean heat production of each unit has been calculated. The results are then presented in the form of equation (3), that is, a straight line graph of $\log_e$ (heat production $\times 10^{13}$) against depth (Fig. 2). The slope of the best fit straight line gives $D = 3.5 \pm 1$ km (2σ).

Considering the scatter of the original data, the fit of the mean values of heat production to the best straight line is remarkably good. It is, however, apparent from Fig. 2 that the value of D is to some extent controlled by the surprisingly low value of mean heat production in the fourth unit. This low value is a result of the relative depletion of uranium with respect to thorium in the basal unit, which in turn reflects the fact that it is only at this structural level that tonalite predominates over the other, more fractionated, granitoid lithologies. It is, however, difficult to further evaluate this low value of mean heat production because the rocks exposed in the basal unit crop out in a smaller area than those of the other three units; and it is not known how typical the lithologies in the fourth unit are of this structural level in the Zentralgneis.

Nonetheless, despite these geological ambiguities concerning the value of mean heat production in the basal kilometre, the scatter of the values of $\log_e$ (mean heat production $\times 10^{13}$) about the best straight line (Fig. 2) is within the assigned experimental errors. Consequently, the distribution of heat production with depth in the Zentralgneis seems to be consistent with an exponential model (equation (2)) in which $D = 3.5 \pm 1$ km, and $A(0) = 15.7 \times 10^{-13}$ calorie $\text{cm}^3 \text{s}^{-1}$.

Other workers have reported results obtained from both heat flow^{1,2} and direct measurement techniques⁵, which are consistent with $D = 9$ –10 km. Roy *et al.*⁶, however, suggested that in stable continental areas, D may be approximately 7.5 km. All these values are significantly greater than the value of D reported here.

Although the Zentralgneis has been deformed and metamorphosed to amphibolite facies, recent geochemical work (C. J. Hawkesworth, unpublished) and the preservation of premetamorphic Rb/Sr whole rock ages⁷, suggests that on a large scale a primary igneous distribution of elements is being observed. Consequently, the low value of D cannot be explained by late upward concentration of the radioactive elements during metamorphism.

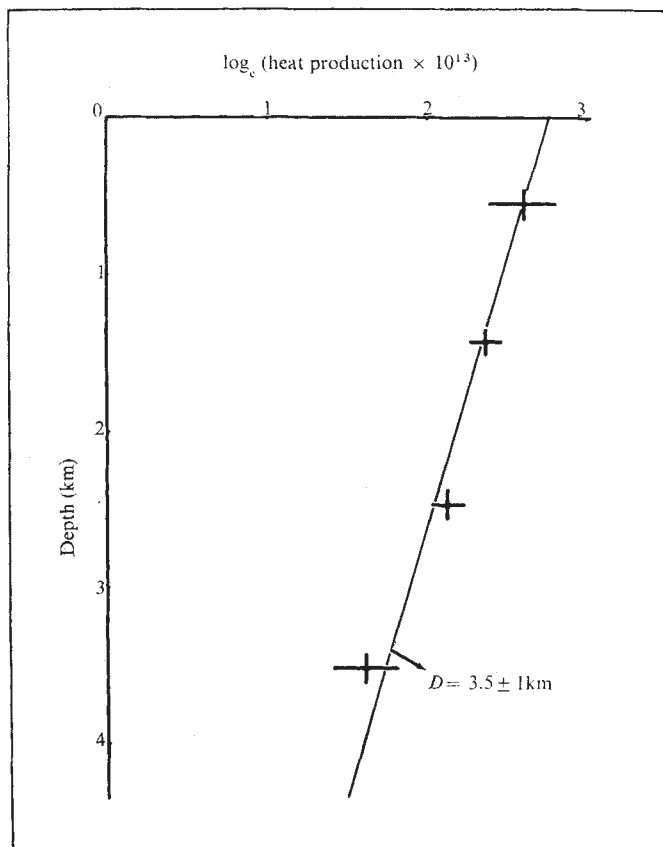


Fig. 2 Distribution of the values of $\log_e$ (mean heat production $\times 10^{13}$) for each of the four horizontal units, with depth in kilometres below the top of the basement complex. The values of mean heat production were calculated from the data in Fig. 1.

Furthermore, this value of D measured at the surface is unlikely to be true for all depths in the present Alpine crust. Recent geophysical work^{8,9} suggests not only that the base of the crust below the Tauern Window is molten but also that below a depth of 10 km there is a large granitic body of assumed Alpine age. This will almost certainly have redistributed the radioactive elements in the lower crust, perhaps with another value of D .

Finally, the results presented here illustrate that the rate of decrease of heat production with depth may be more variable than was initially anticipated. The value of D presumably varies with the regional conditions under which fractionation and intrusion of magma took place. At this time, however, it is not possible to decide whether an exponential decrease of heat production with depth is the direct consequence of fractional crystallisation processes, or whether other factors, such as the effect of the gravitational field on the distribution of large ionic complexes¹⁰, also contribute significantly.

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C. J. HAWKESWORTH

University of Oxford,
Department of Geology and Mineralogy,
Parks Road,
Oxford, OX1 2PR, UK

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Iron and manganese encrustations in Recent sediments

CORES taken from the north-eastern Mediterranean (Fig. 1) during cruise 4/72 of the RRV *Shackleton*, using a Lehigh 4-inch hydroplastic gravity corer, contain layered organic structures encrusted with either manganese or iron minerals (Table 1, Fig. 2).

To our knowledge these encrustations have not previously been reported from seafloor sediments. The bulk of the manganese deposits, found in layers in the top 5–25 cm of the cores, occur on tubes, which we consider to be the agglutinating benthonic foraminiferan *Rhabdammina* (Fig. 3a and b). The deposit is also found on small branching rods (Fig. 3d) which are believed to be of organic origin but have not yet been identified. Smaller deposits are occasionally found on the shells of some of the pteropods and planktonic foraminifera (Fig. 3c); large tubes (Fig. 3e) are thought to be the encrusted linings of burrows. The iron deposits, present in layers beneath the manganese deposits, occur as encrustations on what seem to be mineralogically altered *Rhabdammina* tubes and, rarely, on planktonic foraminifera; no iron equivalent of the branching rods or large tubes has been found. When a core is

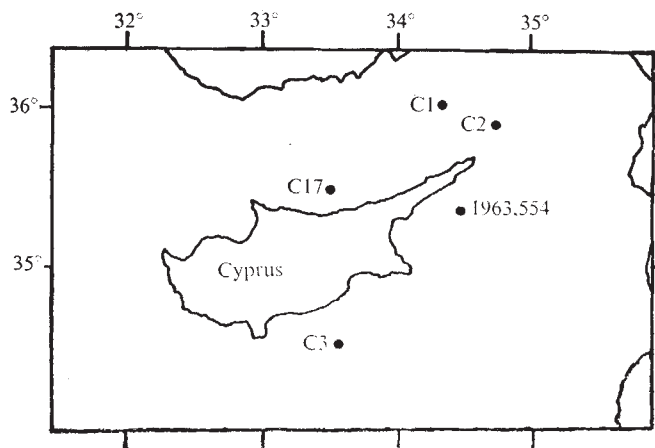


Fig. 1 Location of cores, C. C1, BM1973,0,1; C2, BM1973, 0,2; C3, BM1973,0,3; C17, BM1973,0,17. 1963,544 is a British Museum valve lead sample.

sectioned the manganese deposits appear as black streaks, and the iron-encrusted tubes can be seen as ochre coloured layers. Both types of encrustation were separated from the sediment by washing carefully with hydrogen peroxide (10 vol.), which disaggregates the clay minerals. The manganese encrustation was removed from the smaller tubes with a solution of 1 M hydroxylamine hydrochloride. This exposed a tube similar to the encrusted *Rhabdammina* found in the top 0–5 cm of the cores. The iron encrustation was removed with 1 M hydrochloric acid, leaving a

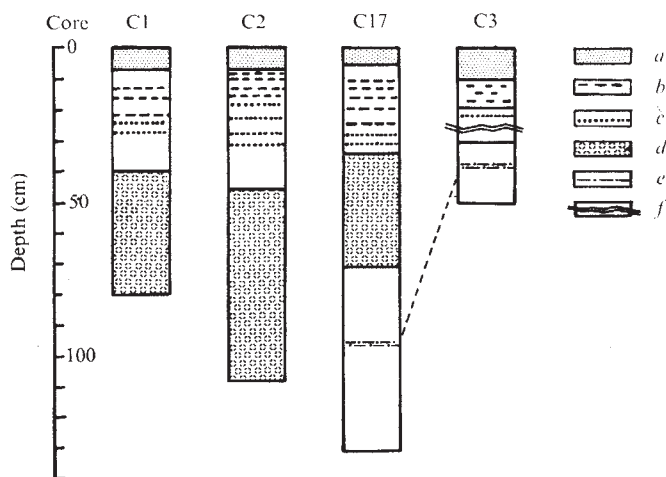


Fig. 2 Sedimentology of the cores. *a*, Orange-yellow ooze; *b*, layer of black manganese-encrusted tubes in light brown ooze; *c*, layer of brown iron-encrusted tubes in light brown ooze; *d*, grey ooze; *e*, light green layer in green-grey ooze (containing no benthonic foraminifera, and possibly representing a sapropel). Cooler water planktonic foraminifera occur below this level. *f*, position of hiatus.

siliceous tube similar to that produced when unencrusted *Rhabdammina* were similarly treated.

Examination by X-ray diffraction of the material rich in manganese showed that it was composed mainly of todorokite, a mineral commonly present in manganese nodules¹. This analysis was confirmed by X-ray powder photography. The structural spacings which we have measured differ slightly from those previously reported², possibly because of the greater amount of magnesium in the structure. The nature of the material rich in iron is not known, but it is amorphous to X rays and is probably a hydroxide.

The faunal content of the cores indicates that they are post glacial. The planktonic foraminiferal population indicates a warm temperature and is dominated by *Globigerinoides ruber* (d'Orbigny), which makes up 60–90% of the samples larger than 250 μ m. It is associated with many gastropods and pteropods, which indicate postglacial sediments in this region^{3,4}. In core C17 below 100 cm, and in core C3 below 45 cm, the occurrence of the foraminifera

feral species *Globorotalia inflata* (d'Orbigny) and *Globigerina pachyderma* (Ehrenberg) provide evidence of cooler conditions. The sediments in core C3 seem to have been disturbed. The distinction between the layers of manganese-rich and iron-rich tubes is less apparent in this core, and the grey ooze section is missing (Fig. 2). A light green layer is present in green-grey ooze at 95 cm in core C17 and is also seen at 27 cm in core C3; this suggests that there is a hiatus somewhere between 20 and 30 cm in core C3, although no physical break is visible in the longitudinally sectioned core. The sedimentation rate in core C17, assuming that a date of 10,000 yr BP (ref. 5) represents the commencement of the postglacial period, is at least 13 cm per 1,000 yr. This implies that the manganese-rich horizons have an age range of about 770 to 2,000 yr BP.

Table 1 Chemical analyses of *Rhabdammina* tubes

	Unencrusted	Manganese-rich encrustations		Iron-rich encrustations	
Core No.	C2	C3	C3*	C1	C2
Depth (cm)	0–4	15–16	15–16	12.5–14.5	20–21
Acid-soluble fraction weight (%)					
Mn	0.1	35.3	26.9	32.5	0.3
Fe	4.2	1.3	1.4	0.7	30.6
Ca	9.0	4.9	5.9	4.2	3.6
Mg	0.6	3.6	3.2	3.3	1.3
Insoluble SiO ₂	67.8	14.5	19.7	<14.9	11.3

*Globular protuberances.

The *Rhabdammina* tubes and other attendant organic debris seem to produce preferential surfaces for the deposition of manganese and iron, as the encrustations only occur in the *Rhabdammina* layers. The presence of the manganese-rich layers above the iron-rich layers may be related to the migratory processes believed to be responsible for the formation of manganese nodules^{6–8}.

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H. A. BUCKLEY
A. J. EASTON
L. R. JOHNSON

Department of Mineralogy,
British Museum (Natural History),
Cromwell Road,
London SW7 5BD

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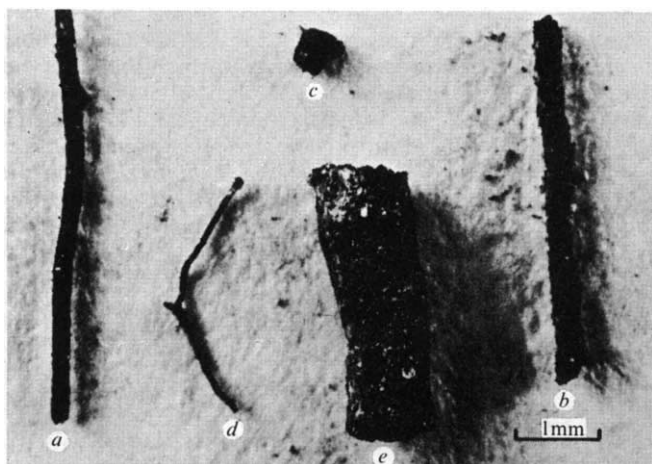


Fig. 3 Manganese encrustations: *a*, Smooth surfaced *Rhabdammina* tubes (typically 0.2–1.2 mm in diameter, and greater than 3 mm long); *b*, *Rhabdammina* tubes with globular protuberances (typically 0.25–0.5 mm diameter, and more than 4 mm long); *c*, planktonic foraminiferan *Globigerinoides ruber* (d'Orbigny); *d*, branching rods (typically 0.075 mm diameter, length variable); *e*, large tube, probably a (?worm) burrow lining (typically 1 mm in diameter and 3 mm long).

Photolysis of CO-NH₃ mixtures and the Martian atmosphere

ONE of the puzzling aspects of the Martian atmosphere is the absence of nitrogen or compounds containing nitrogen¹. Instead, the atmosphere is found to be CO₂ along with smaller amounts of CO and H₂O. (ref. 1). One suggestion is that nitrogen

atoms gain sufficient energy to escape the gravitational field when N_2 undergoes photodissociation with ultraviolet light². Alternatively, nitrogen may be conserved in compounds in the Martian crust³.

We have already noted that although neither CO_2 nor H_2O affected the rate of NH_3 photolysis, CO accelerated the photodecomposition of ammonia, with the formation of a solid product⁴. We have investigated the photolysis of NH_3 in the presence of CO in greater detail not only because of the potential significance to atmospheric photochemistry on Mars but also because of the possibility of photocatalytic reactions of NH_3 and CO on the Martian surface⁵ and in the interstellar medium⁶. These photochemical reactions may also have occurred on the primitive Earth⁷.

Ammonium cyanate has been identified as the major product of the photolysis of gaseous NH_3 -CO mixtures at wavelengths of 206.2 and 184.9 nm. This compound was probably not detected by previous workers because it sublimes readily at room temperature and atmospheric pressure (see refs 8-10). Urea and biurea were isolated in yields that were approximately 10% of the yield of ammonium cyanate and formamide, and the yields of semicarbazide, cyanide and biuret were about 10% of the yield of urea and biurea.

Water-soluble ^{14}C -organic compounds are formed on siliceous substrata which are irradiated in the presence of ^{14}CO , NH_3 and either N_2 or $^{12}CO_2$ diluent gas. For example, 119 nmol of ^{14}C -organics were produced when 0.5 g of vycor glass particles containing 0.28 mmol of adsorbed NH_3 were irradiated for 12 d in the presence of 250 mmol of CO, using an ultraviolet source which had spectral quality and intensity of the sunlight which reaches Mars. This synthesis occurs using an ultraviolet source with a wavelength of approximately 210 nm or greater than 250 nm. Little reaction was observed with dark irradiated samples or when the light source contained only wavelengths longer than 280 nm. Silica gel and volcanic ash shale were also used as substrata for the synthesis. Higher yields were obtained when the irradiations were carried out at $-110^\circ C$. Substrata which were predried at $700^\circ C$ or $400^\circ C$ *in vacuo* were more effective than those which were predried at $150^\circ C$ even though more NH_3 was absorbed by the latter. One factor responsible for these changes may be the level of water adsorbed on the substrata. Addition of water vapour to the gas mixture inhibits, but does not completely prevent, the formation of organic compounds.

Thin layer chromatography (TLC) and autoradiography provided tentative identification of ^{14}C -urea and showed it to be a major product. The identification was substantiated by eluting the suspected ^{14}C -urea spot from the TLC plates, and demonstrating that it was digested by urease. ^{14}C -urea was also detected on substrata which had been predried at $150^\circ C$, but TLC and hydrolysis indicated that formamide was the major product. ^{14}C -formaldehyde has been tentatively identified as a minor product on substrata which had been predried at $700^\circ C$ or $400^\circ C$ by the dimedon derivative procedure (ref. 11, and J. P. Ferris *et al.* not yet published). Using hot water extraction to remove the products from the substrata would have destroyed any ammonium cyanate.

In the experiments which used light with a wavelength of more than 250 nm the reactions were obviously catalysed by wavelengths longer than those absorbed by the gaseous reactants. The absorption coefficient for NH_3 is less than 10^{-4} cm^{-1} at 240 nm¹¹ whereas the longest wavelength at which CO shows any ultraviolet absorption is 206 nm (ref. 12). Thus the reactions are initiated by excitation and/or photolysis of the gas(es) which are adsorbed on the surface, by the longer ultraviolet wavelengths.

The findings discussed here indicate an additional possible mechanism of nitrogen conservation. If NH_3 is outgassed from the Martian crust it would be photolysed and would react with CO, yielding cyanic acid. The cyanic acid could well precipitate as ammonium cyanate which in turn would be converted to urea.

Surface reactions involving CO and NH_3 would also be compatible with conditions existing on Mars. The dust storms known to occur on Mars would expose large amounts of the surface to sunlight. Such dust storms could also effectively bury the particles, thus protecting the organic products from photodestruction.

If NH_3 has been outgassed during the recent history of Mars, it seems highly probable that the molecular analysis experiment on the 1976 Viking mission¹³ will detect nitrogenous compounds, such as urea, in the Martian soil. If the Viking mission obtains evidence suggesting that life exists on Mars, a test for urease¹⁴ may be useful in characterising metabolic processes in later Mars landings.

Both NH_3 and CO occur in interstellar space. CO is the most abundant compound detected in the Sagittarius and Orion nebulae⁶. Cyanic acid and formamide have also been identified in these nebulae⁷ and it is therefore possible that these compounds were produced photochemically from CO and NH_3 . The adsorption of these molecules on the surfaces of particles would increase the probability of reaction even though their concentrations in interstellar space are extremely low. Furthermore, the reactions could make use of the more abundant ultraviolet light at wavelengths of up to 250 nm. Our observation of an increased synthetic rate at low temperatures are also compatible with interstellar conditions. Recent experiments have suggested that the irradiation of silica gel in the presence of CO yields a silica ester of formic acid which may undergo further photochemical reactions with NH_3 to produce urea and formamide. It has also been demonstrated that ultraviolet light with long wavelengths ($> 250 \text{ nm}$) causes the formation of CO_2H and CO_2^- radicals from CO and silica gel¹⁵. The surface radicals produced were unusually stable at low temperatures. Thus the formation of formate ester and/or radicals on interstellar grains could provide stable conditions for subsequent reactions with NH_3 .

There is some disagreement concerning the composition of the atmosphere of the early Earth. A highly reduced CH_4 , NH_3 and H_2O atmosphere is preferred by some whereas others believe that a more oxidised CO_2 , N_2 and H_2O atmosphere (with some more highly reduced compounds) is more likely⁷. In our view any ammonia in the primitive atmosphere would have been rapidly photolysed to N_2 and H_2 . In the presence of CO formed by outgassing or by the photolysis of CO_2 , the NH_3 could, however, have been efficiently converted to cyanate. Some of this cyanate would be hydrolysed back to CO_2 and NH_3 , and some would be thermally converted to urea¹⁶. Some of it, however, may also have effected the formation of pyrimidines¹⁷ and high energy phosphate bonds¹⁸. If these reactions were significant they probably occurred early in the history of the Earth. The rapid rate of the photolysis of ammonia limits the time period in which it would have been present in significant amounts on the early Earth⁴. The surface-dependent syntheses described here would be of questionable importance on the primitive Earth because of their susceptibility to inhibition by water. The reaction yielding formate, formaldehyde and other products^{5,11} seems more likely to have occurred on the terrestrial surface if CO was indeed present.

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J. P. FERRIS,
E. A. WILLIAMS
D. E. NICODEM

Department of Chemistry,
Rensselaer Polytechnic Institute, Troy,
New York 12181

JERRY S. HUBBARD*
GERALD E. VOECKS

Space Sciences Division,
Jet Propulsion Laboratory,
California Institute of Technology,
Pasadena,
California 91103

Received January 8, 1974.

*Present address: Division of Biology, California Institute of Technology, Pasadena, California 91109.

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Crystalline (xanthotoxin)₂.KI₃

STUDIES of the crystal structure of many organic and inorganic tri-iodides have shown that they are salts¹. What happens when a tri-iodide salt forms a complex with a neutral, but polar, organic molecule? Examples, which are formed by related organic molecules, are (coumarin)₄.KI₃ (ref. 2) and (xanthotoxin)₂.KI₃ (ref. 3). (Xanthotoxin is 8-methoxy-3',2':6,7-furocoumarin). The crystals of the first of these substances show one-dimensional disorder⁴ and its structure is not known; we have now determined the crystal structure of (xanthotoxin)₂.KI₃, which is fully ordered, and find that there are potassium cations, tri-iodide anions and neutral xanthotoxin molecules in the crystal. The structure

were obtained: triclinic, $a = 9.59(1) \text{ \AA}$; $b = 11.09(1) \text{ \AA}$; $c = 7.96(1) \text{ \AA}$; $\alpha = 118.1(1)^\circ$; $\beta = 86.7(1)^\circ$; $\gamma = 114.5(1)^\circ$; space group $P\bar{1}$ (according to the structure analysis); $D_m = 2.11 \text{ g cm}^{-3}$, $D_c = 2.11 \text{ g cm}^{-3}$ for 1 formula unit per cell.

Intensities of about 1,900 reflections were measured using a Stoe semi-automatic Weissenberg diffractometer (Mo $K\alpha$, graphite monochromator). Appropriate corrections were made for geometrical and absorption factors. The structure was solved by Patterson and Fourier methods and refined by least squares, using anisotropic temperature factors for all non-hydrogen atoms. The present R factor is 8.2%.

The crystal structure (Fig. 1)⁵ can be conveniently described in terms of extended two-dimensional slices, one xanthotoxin molecule thick, which are bound by $(2\bar{2}0)$ and $(\bar{2}20)$ planes (Fig. 2). The bonding between these slices is by dispersion forces and is appreciably weaker than that within the slices, which results from ionic and dipolar interactions. Each slice is composed of stacks of xanthotoxin molecules, interleaved by columns of alternating cations and anions. The axes of both stacks and columns extend in the $[001]$ direction. Within the xanthotoxin stacks the successive molecules are antiparallel, with an interplanar distance of 3.44 Å. In crystalline xanthotoxin⁸ the stacks contain parallel molecules, with an interplanar distance of 3.58 Å. The shortening in the present compound is presumably because of dipole-dipole interaction between antiparallel molecules, in addition to the normal dispersion forces. There is also an appreciable cation-anion interaction in the $[001]$ direction; each K^+ ion lies at the centre of a parallelogram formed by central and outer iodine atoms of tri-iodide ions above and below it along $[001]$. The distance of K^+ to the outer iodine is 3.53 Å, equal to the shortest $K^+ \cdots I^-$ distance in crystalline potassium iodide⁹, and to the central iodine 3.98 Å (the distance to the second outer iodine is 6.04 Å). The main interaction between stacks and columns is an ion-dipole interaction between K^+ ions and the methoxy and lactonic oxygens of the xanthotoxin molecules, with $K^+ \cdots O$ distances of 2.75 and 2.88 Å respectively. These distances are similar to the $K^+ \cdots O$ distances of 2.76 and 2.80 Å found in potassium hydrogen bis (homophthalate)¹⁰. On the other hand the $C \cdots I$ distances between iodines of the I_3^- ion and the non-polar part of the xanthotoxin molecule, lie in the range 4.10–4.24 Å, showing that these are normal $C-H \cdots I$ contacts.

The dimensions of the xanthotoxin molecule are similar to those found in crystalline xanthotoxin⁸, but the carbon atom of the methoxyl group is less rotated out of the mean plane

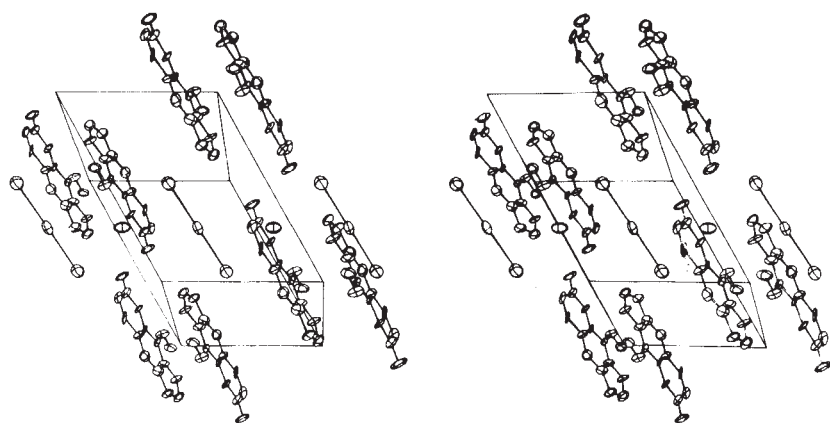


Fig. 1 An ORTEP⁷ stereoview of the unit cell. The origin of the unit cell is at the centre of the parallelepiped. The stacks of xanthotoxin molecules run along the eight edges of the parallelepiped; a pair of xanthotoxin molecules in each of these stacks has been omitted to avoid complicating the diagram. The K^+ and I_3^- ions are both centred at centres of symmetry and the pairs of xanthotoxin molecules shown are related by centres of symmetry. Hydrogen atoms have been omitted.

is thus similar to those of other neutral molecule-salt complexes, such as (succinimide)₂.tetraethylammonium bromide³. Presumably (5,5-diethylbarbituric acid)₂.KI₃ has a similar structure but only a very brief report has appeared⁶.

Strongly dichroic reddish-brown rhombs, somewhat elongated along $[001]$ and showing (100) and (010) faces, were grown from ethanol. The following crystallographic results

of the furocoumarin moiety than in crystalline xanthotoxin (deviations from the mean plane are 0.27 and 0.94 Å respectively). The tri-iodide ion is centrosymmetric, with $d(I-I) = 2.939(2) \text{ \AA}$, a value similar to those found in other structures containing noninteracting I_3^- ions¹.

Thus (xanthotoxin)₂.KI₃ is a neutral molecule-salt complex, with the individual structural elements clearly discernible

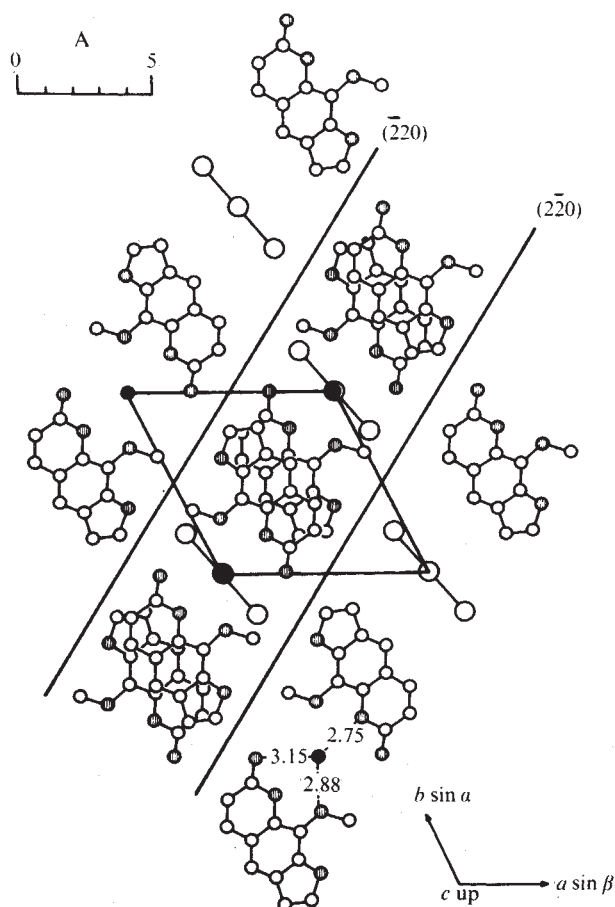


Fig. 2 The crystal structure seen in projection down [001]. An infinite two-dimensional slice, bounded by (220) and $(\bar{2}20)$ planes and extending normal to the page, is shown. The shorter interatomic distances mentioned in the text have been included in the figure. Hydrogen atoms have been omitted. The slices on the edges of the figure each contain only a single layer of structural units so as not to complicate the diagram. Large open circles, iodine atoms; small open circles, carbon atoms; solid circles, potassium atoms; hatched circles, oxygen atoms.

the crystal. The mutual arrangement of these is such as to provide both local neutralisation of charge, the importance of which has been emphasised by Powell and Wait⁵, and an antiferroelectric interaction in the stacks of dipolar xanthotoxin molecules.

M. KAPON
F. H. HERBSTSTEIN

Department of Chemistry,
Technion,
Israel Institute of Technology,
Haifa, Israel

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New vapour deposition technique

THERE are various techniques of vapour deposition for putting down thin films, or thicker coatings and deposits of one material on another^{1,2}, which are of interest to a growing number of industries. The techniques range from chemical vapour deposition at relatively high gas pressure and temperature to physical vapour deposition based on thermal evaporation in a high vacuum for deposition on to hot or cold substrates. In recent years interest has grown in several intermediate techniques in soft vacuum (10^{-3} to 1 torr) which may be physical and/or chemical in nature³⁻⁵. Gas discharges are sometimes used to influence both the physical and chemical aspects of the process—to generate vapour by sputtering, to cause chemical reactions among vapour species, or to encourage bonding to the substrate. Here I outline the basic features of a new 'intermediate' technique which depends simply on evaporation into a heated gas. The aim is to cause the vapour species to diffuse to the substrate while avoiding conditions favouring homogeneous nucleation in the gas phase. Chemical reactions may or may not occur depending on the choice of gas and gas discharges can be employed for substrate cleaning, or other purposes if required.

Homogeneous nucleation within an ambient gas occurs readily at high concentrations of a vapour species and indeed has long been exploited for the manufacture of powders¹ (such as carbon black and pigments). It can readily occur in soft vacuum⁶ and particularly fine powders can be made this way⁷. But its occurrence under vapour deposition conditions is

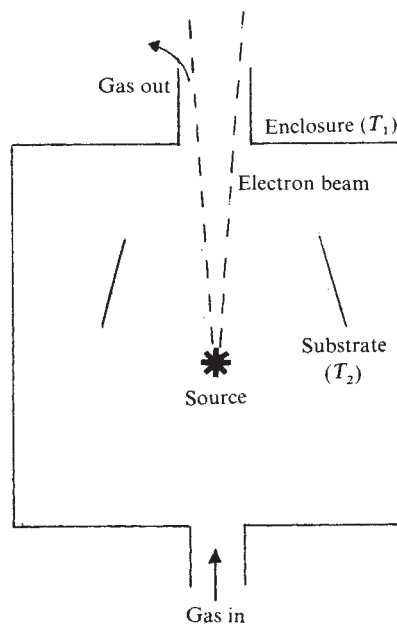


Fig. 1 Schematic diagram of apparatus.

usually undesirable since a poorly bonded granular deposit results. There have been several attempts to construct accurate theoretical models for homogeneous nucleation^{8,9}, though as yet no entirely satisfactory experimental verifications have been made. At least one investigator¹⁰ thinks that the theoretical problem is beyond precise solution at present. But inspection of the theory reveals that the rate of formation of stable clusters should increase with vapour concentration, but decrease as the ambient temperature increases owing to an increase in the critical size for stable nuclei. Lydlin¹¹ showed how to raise the soot limit and consequent growth rate of pyrolytically deposited graphite by mechanical means for reducing the vapour concentration.

The vapour concentration C at any specified coordinates in a given geometry of source and sinks is, from the diffusion equation, proportional to the source strength S and inversely proportional to the diffusion coefficient D (which in turn is

proportional to the product of the average molecular speed c and the mean free path λ). Thus

$$C \propto S/D \propto S/(c\lambda) \propto S/T^{3/2}$$

because of the dependence on temperature (T) of c and λ . Raising the gas temperature will reduce the vapour concentration and increase the size of stable nuclei. So it should be possible to find a range of operating conditions of gas temperature, pressure and rate of evaporation which avoids homogeneous nucleation and precipitates material in molecular form onto the substrates (light gases are favoured by virtue of a larger mean free path).

An experimental investigation of a technique based on these principles was made with apparatus the essential features of which are illustrated in Fig. 1. The heated enclosure was mounted within a soft vacuum chamber (10^{-2} to 1 torr). Electron beams generated by a glow discharge gun¹² were conveniently used for heating purposes. Dense deposits of ceramics (silica, alumina, magnesia, spinel, silicon nitride, titanium carbide and carbon), metals (aluminium, titanium, chromium and molybdenum) and a semiconductor (silicon) have been put down on a variety of substrates including glasses, ceramics, metals and, again, silicon. Several gases were investigated including hydrogen, helium, nitrogen, oxygen and argon and rates of deposition were typically in the range 10^{-2} to 1 mm h⁻¹. At enclosure temperatures T_1 in the range 300° C to 600° C homogeneous nucleation was insignificant at gas pressures of 400/ d mtorr, where d (cm) is the dimension of the enclosure (and therefore equivalent to several tens of mean free paths in a heavy gas such as argon). No investigation of the range of appropriate gas pressures has yet been made, except in the case of hydrogen, for which satisfactory deposits were still obtained at 4000/ d mtorr and this is probably not an upper limit.

The structures of the deposits obtained were essentially similar to those well-known in chemical vapour deposition¹. Depending on the substrate temperature T_2 and the properties of the source material, structures from amorphous through polycrystalline to single crystal were obtained. The bond to the substrate was dependent on chemistry and could sometimes be influenced by the chemical nature of the gas. The texture of deposits was dependent on the smoothness and cleanliness of the substrate surface. Stresses of well known character were found in the deposits, though in a general way the higher the substrate temperature T_2 the lower the grown-in tensile stress.

The advantages of the technique are several. Only roughing pumps are needed, at least for small-scale operations. The transport by diffusion results in good 'throwing power'¹³. The heating of the enclosure degasses the contents and thereafter purity is maintained by the flowing gas which, because of a relatively long and narrow exit orifice, prevents back diffusion of contaminants from outside. On the other hand the gas readily permits an electron beam (of 15–30 keV) to enter though any suitable method for vapourising the source, including sputtering, can be used. Since many of the parameters can be adjusted independently a high degree of process control is possible. The technique is readily adaptable to the deposition of alloys, complex compounds and multilayers and is compatible with other beam techniques for processing materials (such as welding, machining and ion implantation). In a general way it offers a versatile and reproducible method for fabricating special materials.

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R. A. DUGDALE

Materials Development Division,
UKAEA, Harwell, Berkshire, UK

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dsDNA made by RNase-sensitive DNA polymerase from RSV-transformed cells

THE hypothesis that the RNA-directed DNA polymerase (reverse transcriptase) may function in normal cells¹ as part of the machinery for gene amplification is being investigated^{2–8}. Cytoplasmic fractions of normal^{6,7,9,10}, neoplastic^{11–14} and virus-transformed cells^{3,15}, contain RNase-sensitive DNA polymerase activities. Most studies revealed that cellular DNA polymerases differed greatly from the oncornavirus RNA-directed DNA polymerase^{7,9} but the nature of the endogenous template of the cellular DNA polymerases was not described. In view of these findings and reports on the participation of RNA in DNA synthesis by DNA polymerases in bacteria^{16–20}, and mammalian^{21–23} cell systems, we investigated the nature of the template and the role of RNA in the synthesis of DNA by the cytoplasmic RNase-sensitive DNA polymerase activity found in rat cells transformed by the B77 strain of Rous sarcoma virus (R(B77) cells). We found that such preparations contained DNA molecules which served as the template for the RNase-sensitive DNA polymerase activity. We also found that RNA has a function in the synthesis of DNA since nascent DNA molecules, covalently linked to RNA, were synthesised at the very beginning of the reaction.

R(B77) cells, a cell line of rat embryo fibroblasts transformed *in vitro* by the B77 strain of Rous sarcoma virus (RSV), were used^{24,25}. The cells were maintained in culture by weekly transfers. The cells were seeded at a concentration of 5×10^5 cells per plastic Petri dish (diameter 90 mm; Nunc, Denmark) in 10 ml Eagle's medium containing 1 or 2 μ Ci ml⁻¹ of 5-³H-uridine (13.8 Ci mmol⁻¹; Nuclear Research Center, Negev, Israel) or 0.1–0.2 μ Ci ml⁻¹ of 2-¹⁴C-uridine (62 mCi mmol⁻¹; Radiochemical Centre, Amersham, England). The cultures were labelled for 24 h, unless otherwise stated. To label cellular DNA, the medium of R(B77) cells was replaced with fresh Eagle's medium containing 1 μ Ci ml⁻¹ of methyl-³H-thymidine (8.2 mCi mmol⁻¹; Nuclear Research Center, Negev). BUdR was incorporated into cellular DNA by adding 30 μ g ml⁻¹ of BUdR (Sigma) to the medium of R(B77) cells. The cells were labelled with ³H-deoxycytidine (1 μ Ci mmol⁻¹; specific activity, 19.4 Ci mmol⁻¹ Radiochemical Centre) and were collected after 48 h.

High speed pellets (HSP) of cells were prepared as described by Coffin and Temin³. The standard assay for the avian sarcoma RNA-dependent DNA polymerase was according to Temin and Mizutani²⁶, as described previously for purified B77 virions^{15,27}. The DNA polymerase activity in HSP was studied under the same conditions as for the avian sarcoma virus enzyme.

High speed pellet (HSP) preparations from R(B77) cells contained a DNA polymerase activity which was markedly inhibited by RNase treatment before and after initiation of DNA synthesis (Table 1). This indicates that the synthe-

sis of DNA by the HSP DNA polymerase activity is RNA dependent. To rule out the possibility that HSP preparations contain RNase and DNase activities, ^3H -uridine and ^3H -thymidine labelled HSP preparations were incubated with or without exogenous nuclease. It was found that the labelled nucleic acids were not degraded after incubation for 60 min at 37°C (not shown).

Table 1 Effect of RNase on DNA synthesis by HSP DNA polymerase activity

Treatment of enzymatic reaction with RNase*	Time (min)		
	5	15	30
	^3H -TMP incorporated (c.p.m. per 25 μl)		
Untreated	1,310	2,050	2,215
Pretreated for 10 min	160	175	215
Treated at 0 time	390	500	660
Treated at 5 min	1,320	1,420	1,590

* The HSP preparation was divided in four portions: one was left untreated, the second was preincubated with RNase A (50 $\mu\text{g ml}^{-1}$) at 37°C , to the third RNase was added immediately before the initiation of the reaction, and to the fourth, RNase was added 5 min after initiation of DNA synthesis *in vitro*. The standard assay of Temin and Mizutani²⁶ was used. Duplicate samples were removed and the TCA-precipitable radioactivity was determined.

The polymerase activity was also characterised by studying the effect of addition of synthetic polynucleotides (Table 2), which can help to distinguish between cellular DNA polymerase and the RNA-directed DNA polymerase of oncornaviruses²⁸. Addition of poly(rA)-oligo(dT) to HSP preparations only slightly stimulated incorporation of ^3H -TMP into DNA whereas it markedly stimulated the RNA-directed DNA polymerase of RSV and poly(dA)-poly(dT) stimulated ^3H -TMP incorporation by both enzymatic activities to the same level. In this respect, the HSP RNase-sensitive DNA polymerase resembles other DNA polymerases present in eukaryotic cells^{7,8}.

Analysis by zonal centrifugation of the DNA molecules synthesised *in vitro* by the HSP RNase-sensitive DNA polymerase showed that the product banded in the sucrose gradient (15–65% (w/v) for 3 h at 45,000 r.p.m. using rotor SW50.1 of the Beckman ultracentrifuge) with a sedimentation coefficient of about 10S. Analysis on a sucrose gradient of the HSP DNA synthesised *in vivo* showed DNA molecules sedimenting to the bottom of the sucrose gradient and two bands of labelled DNA, of which the major peak had a sedimentation constant of about 10S (not shown).

The DNA synthesised by HSP DNA polymerase activity during 1 min and 20 min incubation was analysed by chromatography on hydroxylapatite columns (Fig. 1a). The DNA synthesised *in vitro* was eluted from the hydroxylapatite columns by 0.25 M phosphate. Labelled DNA prepared from R(B77) cells had the same elution profile as the DNA synthesised *in vitro*. Cellular DNA was eluted before the ^{14}C -labelled HSV DNA (0.27 M phosphate) at the position of nuclear DNA. This demonstrated that the DNA molecules synthesised *in vitro* are double-stranded and resemble nuclear DNA in their elution properties. There was no difference between the DNA molecules synthesised during a short (1 min) or a long (20 min) period.

To study the effect of actinomycin D on DNA synthesis by the HSP DNA polymerase, actinomycin D (250 $\mu\text{g ml}^{-1}$) was added to the standard reaction mixture which was then incubated for 20 min at 37°C . After extraction the DNA product was compared with DNA synthesised by HSP in the absence of inhibitor. Actinomycin D inhibited DNA synthesis by 80% compared with the untreated control. The DNA product synthesised by the HSP treated with actinomycin D was analysed by chromatography on a hydroxylapatite column. It was found (Fig. 1b) that this DNA was eluted by 0.25 M phosphate, similarly to the cellular double-stranded DNA (dsDNA) molecules. The HSP DNA polymerase activity differs from Rous

sarcoma virus reverse transcriptase, in respect of the effect of actinomycin D (Fig. 1c). In the presence of actinomycin D, the viral enzymatic activity synthesises single-stranded DNA (ssDNA) molecules which are eluted from hydroxylapatite by 0.1 M phosphate.

High speed pellets were prepared from R(B77) cells, labelled or unlabelled for 48 h with ^3H -deoxycytidine and incubated in the presence or absence of BUdR. The DNA was extracted with phenol and banded in CsCl gradients (Fig. 2a). Untreated DNA banded at a density of 1.70 g cm^{-3} whereas the BUdR-containing DNA banded at the density of 1.74 g cm^{-3} (Fig. 2d). Samples from two unlabelled HSP preparations (prepared from cells grown in the absence or presence of BUdR) were incubated *in vitro* for 60 s under conditions of DNA synthesis with the four deoxyribonucleoside triphosphates (including ^3H -TTP). The DNA was extracted and banded in CsCl gradients. The DNA synthesised *in vitro* in the HSP from untreated cells banded at a density of 1.70 g cm^{-3} (Fig. 2b), the density of cellular DNA. The radioactive DNA synthesised *in vitro* in the presence of BUdR-containing DNA (HSP from BUdR-treated cells) partly banded at the density of the BUdR-labelled DNA and part was shifted to a smaller density (Fig. 2e). Longer incubation of the BUdR-containing DNA from HSP (5 min) resulted in a further shift to a lower density (Fig. 2f), similar to untreated DNA (Fig. 2c). These results demonstrate that the DNA polymerase activity present in the HSP utilised the DNA molecules present in the HSP as the template. The complete inhibition of this reaction by RNase suggests the involvement of RNA as a primer or an intermediate during DNA synthesis by the HSP DNA polymerase.

High speed pellet preparations were incubated under conditions of DNA synthesis and the processes were stopped at 1 min and 5 min after the initiation of the enzymatic reaction. The ^3H -TMP labelled DNA synthesised *in vitro* was extracted with phenol, precipitated by ethanol, resuspended in Tris-EDTA buffer, and divided into three: one portion was left as native DNA, the second was heat denatured, and the third was denatured by alkali, and each sample was banded in a Cs_2SO_4 gradient. Most of the native DNA product banded at the density of dsDNA (1.42 g cm^{-3}) but some radioactive DNA appeared at higher densities (1.58–1.69 g cm^{-3}) which are those of DNA molecules complexed or associated with RNA. The amount of DNA in this region, slightly increased after heat denaturation, but the radioactivity disappeared after treatment with alkali. Similar results were found with the DNA synthesised during a reaction period of 5 min. We then investigated the nature of the DNA molecules synthesised during the shorter reaction times, 7, 15, and 30 s (Fig. 3). Analysis of the 7-s DNA product (Fig. 3a) revealed DNA molecules banding throughout the gradient, mostly at the densities of dsDNA (1.42 g cm^{-3}) and RNA (1.69 g cm^{-3}). The DNA molecules which initially banded at the density of RNA (1.69 g cm^{-3}), most probably DNA:RNA hybrid molecules, were not detected in Cs_2SO_4 after heat denaturation or alkali treatment (Fig. 3b and c). Analysis of the DNA molecules synthesised during 15 or 30 s revealed that the total amount of native heavy DNA (which banded at 1.69 g cm^{-3}) was greatly decreased (Fig. 3d and g). After heat denaturation, however, the amount of radioactive DNA which appeared in the Cs_2SO_4 gradient between the DNA and RNA, most probably DNA–RNA complexes (1.58–1.69 g cm^{-3}) also increased (Fig. 3e). The same findings were obtained with the DNA product of a 30-s reaction (Fig. 3g and 3h). These reaction products disappeared on treatment with alkali after which the labelled molecules accumulated in the position of ssDNA (Fig. 3f and i). In all nine experiments carried out there was a significant increase of radioactivity in the region of RNA–DNA complexes after heat denaturation which disappeared after alkali treatment. This

indicates that part of the newly synthesised DNA molecules may be linked to RNA molecules. After a short incubation (7 s) the DNA molecules may have been synthesised on RNA molecules, which serve as templates or initiators. The labelled native DNA molecules, band at the density of dsDNA (1.42 g cm^{-3}) although some of it might still be

covalently bonded to RNA and can be detected after heat denaturation.

The presence of an RNase-sensitive DNA polymerase activity in normal and transformed cells, which differ from the oncornavirus RNA-directed DNA polymerase, has recently been reported^{3,6,7,9,10,15}. Our study confirms the presence of an RNase-sensitive DNA polymerase activity in R(B77) cells. The novel finding of this study is the demonstration that the HSP of R(B77) cells contains 10S DNA molecules of cellular origin which act as the template for this enzymatic activity and that RNA molecules also participate in the synthesis of DNA.

Table 2 Effect of poly(A)-oligo(dT) and poly(dA)-poly(dT) on DNA synthesis by the RNase-sensitive DNA polymerase activity from transformed R(B77) cells and the B77 Sarcoma virus reverse transcriptase activity

Pretreatment† of HSP	Polymer added	³ H-TMP incorporated (c.p.m.)*	
		R(B77) enzyme	Viral‡ enzyme
Exp. 1	Buffer	1,887	1,871
	RNase	365	ND§
	Buffer	2,310	126,540
Exp. 2	Buffer	1,145	5,551
	Poly(dA)-poly(dT)	2,193	10,245

* The standard assay for oncornavirus RNA-dependent DNA polymerase and HSP DNA polymerase activity was according to Temin and Mizutani²⁶. To each reaction mixture, 0.01 $A_{260 \text{ nm}}$ units of poly(dA)-poly(dT) or poly(A)-oligo(dT) (Collaborative Research, Waltham, Massachusetts) were added.

† The enzyme preparations were incubated for 15 min at 37°C with RNase ($50 \mu\text{g ml}^{-1}$) or buffer before the addition of the standard reaction mixture. ‡ B77 virus treated with NP-40. § Not done.

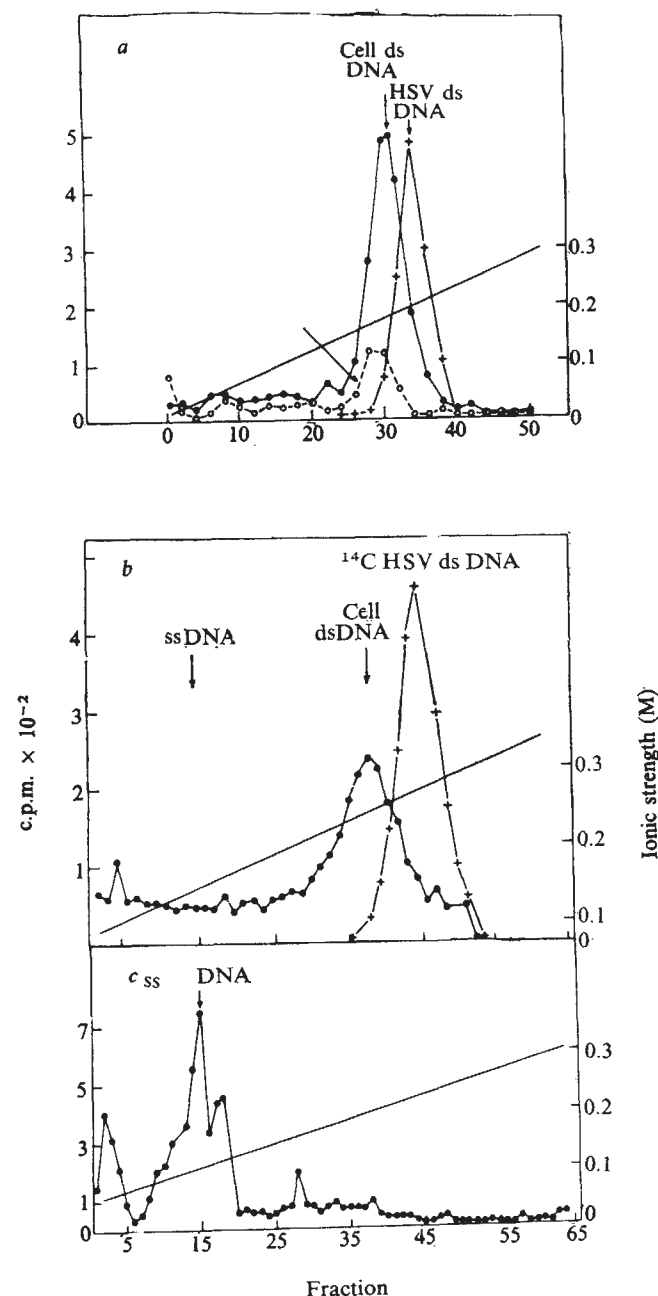


Fig. 1 Hydroxylapatite chromatography of DNA molecules synthesised by HSP DNA polymerase and Rous sarcoma virus reverse transcriptase in the absence and presence of actinomycin D. *a*, HSP was incubated with the standard reaction mixture and samples were removed at 1 and 20 min, treated with 1% SDS, the DNA was extracted with phenol and characterised by chromatography on hydroxylapatite columns (Bio-gel HTP; Bio-Rad Laboratory, Richmond, California), eluted with a linear gradient ranging from 0.05 M to 0.35 M of sodium phosphate (total volume 100 ml) and collected in about 40 fractions. TCA precipitable radioactivity in each fraction was collected on filters and counted in a liquid scintillation counter. Purified herpes simplex virus (HSV) DNA, labelled with ^{14}C -thymidine as well as BSC₁ DNA and HeLa DNA were used as markers. The results of three columns are combined in the graph. O, DNA molecules synthesised by a HSP preparation during 1 min; ●, DNA molecules synthesised by a HSP preparation during 20 min; +, ^{14}C -HSV DNA marker. The position of cell DNA is indicated by the arrow. *b*, An HSP preparation

The DNA polymerase activity present in HSP resembles the oncornavirus-associated reverse transcriptase in its sensitivity to RNase and in its ionic requirements^{3,15}. Nevertheless, marked differences were found between the two enzyme activities in agreement with the findings of Bobrow *et al.*⁹ and Kang and Temin⁷. We demonstrated that (a) the HSP enzyme was only slightly stimulated by poly(rA)-oligo(dT) which stimulates the viral enzyme, whereas poly(dA)-poly(dT) had the same stimulatory effect on both HSP and the viral enzymes; (b) the main product of the HSP RNase-sensitive DNA polymerase is dsDNA even after a very short reaction; (c) although actinomycin D inhibits both enzymes, only with the viral enzyme is there an accumulation of ssDNA molecules (Fig. 2); and (d) the endogenous template for the cellular RNase-sensitive DNA polymerase is DNA (Fig. 3), whereas for the viral enzyme, it is RNA. These results show that the HSP DNA polymerase activity is an RNA-dependent DNA polymerase, which utilises the cellular dsDNA molecules present in HSP as template.

What is the function of RNA during the synthesis of DNA? Analysis in Cs_2SO_4 density gradients of the DNA

was incubated with actinomycin D ($250 \mu\text{g ml}^{-1}$) and the ^3H -TMP labelled DNA was phenol extracted and chromatographed on a hydroxylapatite column. ^{14}C -HSV DNA and cellular dsDNA served as markers. ●, ^3H -DNA synthesised by HSP enzymatic activity in presence of actinomycin D; +, ^{14}C -HSV DNA marker. Cell DNA eluted identically to HSP DNA. *c*, Viral reverse transcriptase activity. Purified B77 virions were disrupted with Nonidet P-40 and incubated *in vitro* under conditions of DNA synthesis by the viral reverse transcriptase, actinomycin D ($250 \mu\text{g ml}^{-1}$) was added and the ^3H -DNA molecules synthesised during 20 min were extracted by phenol and analysed by chromatography on a hydroxylapatite column^{27,30} (●).

products resulting from very short reactions revealed radioactive DNA banding at the density of RNA (1.69 g cm^{-3}) as well as labelled DNA molecules which banded as dsDNA. Radioactive DNA also banded at densities ranging from that of RNA to DNA ($1.69\text{--}1.58 \text{ g cm}^{-3}$) suggesting that the DNA molecules are attached to RNA molecules. The amount of label which appeared in these regions, increased after heat denaturation and shifted to the density of ssDNA after alkali treatment, proving that they are DNA molecules linked covalently to RNA chains (Fig. 3) which initially banded together with the dsDNA molecules. It is possible that the RNA-DNA complexes described here resemble the RNA-DNA pieces described

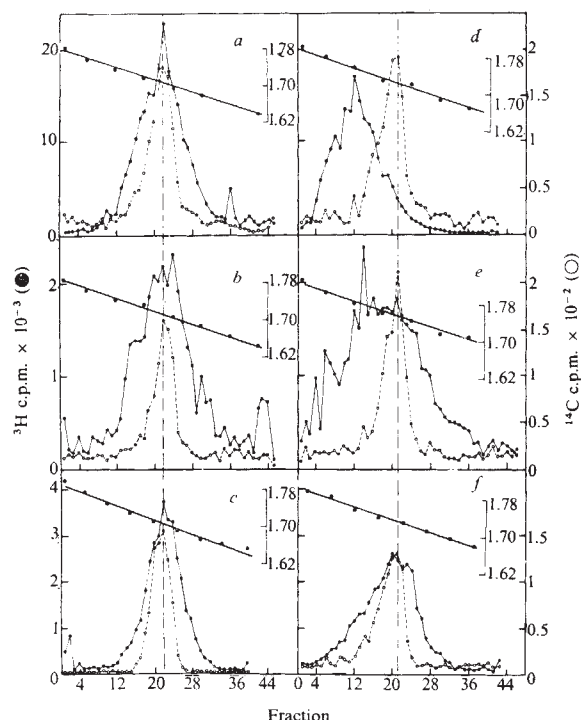


Fig. 2 HSP DNA as template for the endogenous RNase-sensitive DNA polymerase. Twelve Roux bottles containing R(B77) cells were incubated for 48 h in the presence or absence of BUdR (5-bromo-2'-deoxyuridine). One Roux bottle from each group was labelled with $1 \mu\text{Ci ml}^{-1}$ of ^3H -deoxycytidine. After incubation, HSP was prepared. Unlabelled BUdR-treated and control HSP preparations were incubated for 1 min and 5 min with the reaction mixture, and the reactions were stopped by addition of SDS to final concentrations of 2%, 0.5% Baycovin, and $50 \mu\text{g}$ of yeast DNA. The nucleic acids from all preparations were extracted according to the Hirt method, modified by Kang and Temin¹⁰. The alcohol precipitates and marker ^{14}C -thymidine labelled DNA from BSC₁ cells were added to 8 ml of a CsCl solution and the mixtures centrifuged for 60 h at 35,000 r.p.m. in the Beckman 50 Ti rotor at 22°C . The gradients were collected and the radioactive TCA precipitates counted. Densities were determined by weighing $100 \mu\text{g}$ samples (●). a, R(B77) cells were labelled with ^3H -deoxycytidine for 24 h after which the DNA was extracted (●). ^{14}C -thymidine labelled R(B77) nuclear DNA was used as a marker (○). The DNA preparations were mixed and analysed by centrifugation in a CsCl gradient. b and c, HSP preparations were prepared and incubated *in vitro* under conditions of DNA synthesis in the presence of ^3H -TMP. The ^3H -DNA was extracted with phenol after 1 min (b) and 5 min (c) reaction periods. The ^3H -TMP labelled DNA (●) was mixed with ^{14}C -thymidine labelled BSC₁ dsDNA (○) and centrifuged in CsCl gradients. d, R(B77) cells were incubated with BUdR and ^3H -deoxycytidine for 24 h. The DNA was extracted (●) and mixed with ^{14}C -thymidine labelled DNA (○) and centrifuged in a CsCl gradient. e and f, HSP preparations were prepared from R(B77) cells incubated for 24 h with BUdR. The HSP was incubated for 1 min (e) and 5 min (f) under conditions of DNA synthesis in the presence of ^3H -TTP. The DNA was extracted with phenol (●) mixed with ^{14}C -DNA (○) and centrifuged in gradients.

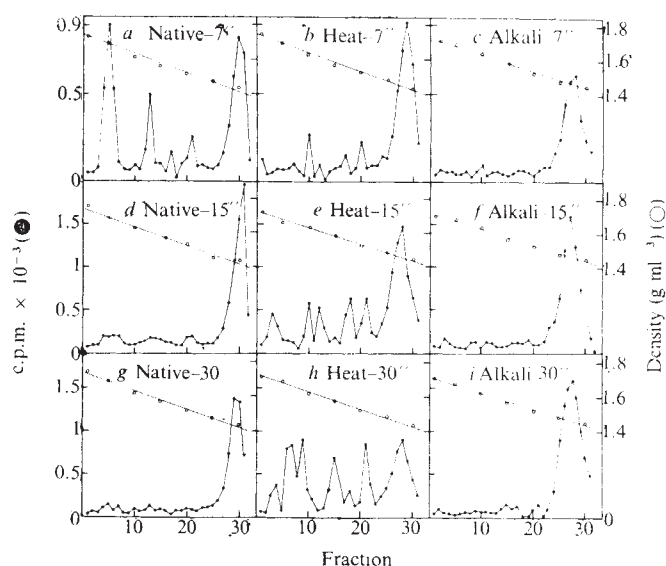


Fig. 3 Characterisation of the DNA molecules synthesised by HSP DNA polymerase activity. HSP was incubated at 38°C for 7, 15, and 30 s with the standard reaction mixture. The reaction was stopped by adding SDS, and the DNA products were purified as described in the legend for Fig. 2. The DNA samples were resuspended in Tris-EDTA buffer and each DNA preparation was divided into three: one was left untreated (a, d, and g), one sample of each was boiled for 10 min and chilled immediately (b, e, and h), and the third sample was treated with 0.25 M NaOH for 10 min and then neutralised by HCl in ice (c, f, and i). All nine DNA preparations were mixed with Cs_2SO_4 to a final volume of 6 ml and centrifuged at 45,000 r.p.m. for 64 h at 22°C in the 50 Ti rotor of the Beckman centrifuge. The gradients were fractionated dropwise, the densities determined by weighing $100 \mu\text{l}$ of the indicated samples, and the TCA radioactive precipitates were counted. ●, Radioactive DNA; ○, density.

by Sugino, Hirose, and Okazaki¹⁸. The participation of RNA in DNA synthesis has also been suggested for mammalian cells²³ and for polyoma virus²⁹. The presence of labelled DNA molecules after 7 s of DNA synthesis which band at a density of RNA (1.69 g cm^{-3}) (Fig. 3a) and disappear after heat denaturation (Fig. 3b) may indicate DNA synthesis on an RNA template, an activity ascribed to oncornavirus reverse transcriptase²⁶. It is possible that low levels of reverse transcriptase activity may be detected only after a very short reaction period, whereas the major DNA polymerase activity in HSP of R(B77) cells is a DNA polymerase activity which requires RNA for its function, most probably as an initiator and not as a template. The role of this enzyme and its DNA template in the R(B77) transformed cell is still to be studied.

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MOSHE KOTLER
OSNAT HASPEL
YECHIEL BECKER

Laboratory for Molecular Virology,
Hebrew University-Hadassah Medical School,
Jerusalem, Israel

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from ³H-UTP after incubation in various modifications of the standard assay medium (see legend to Tables 1 and 4). The reaction product satisfied the standard tests for RNA (Table 2). The reaction clearly depended on the presence of RNA, the amount of incorporation increasing linearly to a maximum as the concentration was increased (Fig. 1). Magnesium ions were also necessary, the optimum concentration of MgCl₂ being 12 mM. Only the four ribonucleoside triphosphates (NTPs) and not the corresponding deoxyribonucleoside triphosphates (dNTPs) could act as substrates. With enzyme from adult rats the omission of any one NTP from the medium markedly reduced incorporation (Table 1a) indicating that when they were all present the product was mainly heteropolymeric RNA. By contrast, the fraction from newborn rat readily synthesised RNA when provided with only a single NTP substrate (Table 1b): presumably this was

Table 1 Characterisation of the reaction in the presence of four ribonucleoside triphosphates

a Assay mixture (μg)		³ H-UMP incorporated (pmol)
Experiment 1	Complete	11.0
	+DNase	10 9.3
	+Actinomycin D	2 8.1
	+RNase (+RNA)	10 1.7
	(-RNA)	10 0.5
	+(NH ₄) ₂ SO ₄ (0.32 M)	3.4
	+(NH ₄) ₂ SO ₄ (0.41 M)	1.1
	-RNA	2.1
Experiment 2	Complete	8.3
	-ATP	3.8
	-GTP	4.4
	-CTP	5.5
	-MgCl ₂	0.5
	-RNA	2.0
b		³ H-NMP incorporated (pmol)
Experiment 1	Complete (³ H-UTP)	23.9
	+DNase	10 22.7
	+Actinomycin D	2 23.6
	+RNase (+RNA)	10 4.7
	(-RNA)	10 0.5
	+(NH ₄) ₂ SO ₄ (0.41M)	7.4
	-RNA	7.7
Experiment 2	Complete (³ H-UTP)	32.8
	-3NTP	25.2
	-RNA	6.0
	Complete (³ H-ATP)	22.9
	-3NTP	20.3
	-RNA	6.1
	Complete (³ H-GTP)	23.2
	-3NTP	15.3
	-RNA	6.0

Where indicated 10 μg of DNase, 10 μg of RNase or 2 μg of actinomycin D was added, and one of the four ribonucleoside triphosphates was omitted. 3 NTP indicates the three ribonucleoside triphosphates other than the tritiated one. *a*, Enzyme and RNA were from adult brain; *b*, enzyme and RNA were from newborn brain (1-d-old). The standard reaction mixture had a total volume of 0.25 ml and contained 22 μmol Tris-HCl (pH 7.4), 3.0 μmol MgCl₂, 4.5 nmol 2-mercaptoethanol, 0.2 μmol each of ATP, CTP, GTP and ³H-UTP (4 × 10⁶ c.p.m. per 0.2 μmol): when assaying adult brain there was 80 μg enzyme protein and 10 μg template RNA; for newborn brain the quantities were 34 μg and 15 μg respectively. The reaction mixture was incubated at 37° C for 10 min for adult enzyme and 20 minutes for newborn enzyme, unless otherwise indicated. The reaction was stopped by addition of pyrophosphate and trichloroacetic acid. Precipitates were collected on membrane filters, washed eight times with cold 5% trichloroacetic acid, and dried according to standard procedures¹. In some experiments the reaction was stopped by blowing aliquots of the assay mixture on filter paper treated with pyrophosphate which was then washed for 2 h in 5% cold trichloroacetic acid according to the method of Bollum¹⁸. The radioactivities of the dried membrane filters or filter papers were determined in 10 ml of toluene scintillator containing 0.5% (w/v) of 2,5-diphenyloxazole with a Beckman LS 100 liquid-scintillation spectrometer. The radioactivity for zero time was subtracted from each value.

RNA-dependent RNA synthesis in rat brain

RIBONUCLEIC acid-dependent RNA polymerase (replicase) was first isolated from *Escherichia coli* infected with RNA bacteriophage Qβ which has specific template requirements¹. Such an enzyme has been described in tumour cells and several types of mammalian cells infected with RNA viruses²⁻⁵.

It has been reported that in the normal mammalian cell polyribonucleotides are synthesised by a nontranscriptive process and enzymes which polymerise ATP to poly(A) have been found⁶⁻⁸. Ribonucleoprotein particles isolated from rat liver nuclei polymerise ATP, UTP, GTP and CTP to the corresponding ribohomopolymers⁹. These reactions have been suggested to be post-transcriptional additions to heterogeneous nuclear RNA. The microsome fraction of rat liver also incorporates the four ribonucleotides into polyribonucleotides a reaction which is stimulated by RNA but not DNA¹⁰. We report here the isolation of an enzyme fraction from rat brain which can synthesise RNA¹¹, both as a heteropolymer or a homopolymer, only when provided with a suitable RNA template or primer.

The enzyme fraction was isolated from rat brain (see legend to Table 2) and its RNA polymerase activity was measured by the radioactivity incorporated into acid-insoluble product

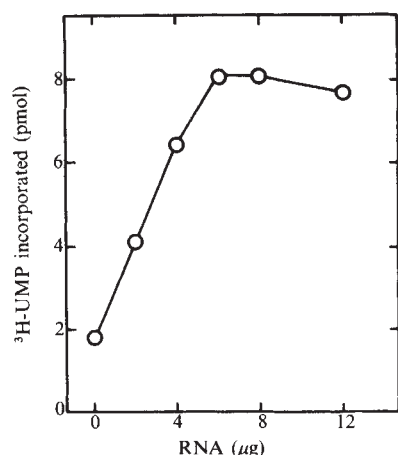


Fig. 1 Dependence of incorporation on concentration of template RNA. The composition of the incubation mixture was the same as described in Table 1 except for the RNA concentration, which varied. RNA and enzyme were from adult rat brain. Incubation was for 10 min.

nearly all homopolymer. When this homopolymer synthesis was investigated it was found, as reported for many other similar systems, that uptake of ³H-UTP was inhibited by the presence of CTP or GTP (Table 4). It is therefore probable that with enzyme from newborn rats presented with all four NTPs nearly all the product was heteropolymer and *a fortiori* for the adult. It was clear that heteropolymer synthesis could be studied more easily using the adult enzyme fraction and homopolymer synthesis using the newborn and we did this, further improving the differentiation by using different concentrations of MgCl₂ in the reaction mixtures.

When the added RNA was omitted from the standard assay medium the incorporation fell to about one quarter. Most of this residual activity could be abolished by the addition of RNase which indicates that this also was mainly RNA-dependent RNA synthesis. The reaction was little affected by the addition of 10 μg of DNase and was inhibited by ammonium sulphate at concentrations which augment DNA-dependent RNA synthesis^{13,14} (Table 1). Two micrograms of actinomycin D had no effect. The effectiveness of various nucleic acids as templates was examined by substituting them for the rat brain RNA in the medium (Table 3). Only RNA was effective: DNA from calf thymus produced very little result. RNAs from Qβ, MS2 or GA phages, representatives of different groups of viral RNA, were ineffective and so were tRNA and rRNA from *E. coli* and TMV RNA. Of the homopolymers, poly(A), poly(G), poly(C) and poly(U) only poly(U) had any effect: either the synthesis was occurring by replication or by addition of UMP only to UMP terminals. Rat liver RNA was quite effective but markedly less so than the rat brain RNA. The degree of template specificity, that is, the range of effectiveness of the templates, was similar to that of the RNA-dependent RNA polymerases isolated from leukaemia and ascites tumour cells by Haruna *et al.*⁵. In the case of RNA replicase (RNA-dependent RNA polymerase) isolated from *E. coli* infected by Qβ phage, a basis for recognition of an RNA by an RNA replicase has been postulated by Kamen *et al.*¹⁵ and Nishihara *et al.*¹⁶. Analysis of the brain RNA by sucrose gradient centrifugation gave the normal pattern for total cell RNA. Under these heteropolymer-promoting conditions the RNA polymerase in the enzyme fraction isolated from newborn rats had properties qualitatively similar to those for the adult but its activity was about three times higher (see Table 1 and Figs 1 and 2; it should be noted that the concentrations of enzyme and RNA differed from those used for the adult). ³H-UTP was incorporated by the adult enzyme at an almost constant rate for the first 10 min of incubation with the template RNA (Fig. 2). During the next 10 min of incubation

the rate declined. In the absence of added RNA the incorporated radioactivity reached a steady level after ~ 5 min incubation. With enzyme from newborn rat the activity increased linearly with time up to 30 min (Fig. 2). The maximum activity

Table 2 Effects of nuclease and alkaline treatment on the product of the adult enzyme reaction.

Treatment	³ H-UMP incorporated (pmol)
None	9.1
Alkaline hydrolysis (0.3 N KOH; 37° C 18 h)	0.3
RNase 10 μg per 0.25 ml	1.0
DNase 10 μg per 0.25 ml	7.9

Reaction mixtures were as in the legend to Table 1. For the alkaline treatment, after 10 min incubation, sodium dodecyl sulphate and KOH were added to give final concentrations of 0.4% (w/v) and 0.3 N and the mixture was incubated at 37° C for a further 18 h. Ten micrograms of RNase or 10 μg of DNase was added to the incubation mixture after 10 min and incubation continued for a further 10 min. The reaction was terminated by addition of pyrophosphate, 50 μg of carrier albumin and trichloroacetic acid. In the case of KOH addition, sodium dodecyl sulphate was added to avoid the degradation of product RNA by RNase contaminant in the enzyme fraction. Adult and 1-d-old rat brains were used to prepare enzyme fraction. Isolated brains containing cerebrum and cerebellum were frozen and thawed once. The brains were minced and suspended in medium (0.1 M Tris-HCl (pH 7.4), 0.01 M MgCl₂ and 0.5 mM 2-mercaptoethanol) containing 5 μg DNase l⁻¹. The mixture, after homogenisation for 40 s, was stirred at 4° C for 30 min. The homogenate was centrifuged at 12,000g for 30 min and EDTA added to the supernatant to a final concentration of 10 mM. This solution was then centrifuged for 30 min at 12,000g. An equal volume of saturated ammonium sulphate (pH 7.4) was added to the supernatant and incubated at 0° C for 10 min and the 50% saturated ammonium sulphate-precipitable fraction was collected by centrifugation at 9,000g for 10 min and dissolved in medium (0.4 M ammonium sulphate, 9 mM Tris-HCl (pH 7.4), 4.5 mM MgCl₂ and 0.45 mM 2-mercaptoethanol). 0.2 volume of glycerol was added to the medium. This was the enzyme fraction. Its protein concentration was 16–28 mg ml⁻¹ and it was stored at –80° C.

increased with increasing concentrations of enzyme. An explanation for the differences in time courses is that the adult enzyme fraction was contaminated by RNase or some inhibitor.

When presented with a single NTP the enzyme fraction from newborn rats synthesised considerable amounts of homopolymer, UTP and ATP being taken up to higher concentrations than GTP (Table 1b). Tests made to investigate heteropolymer synthesis by adult enzyme were repeated (Table 4) and showed that poly(U) synthesis was also independent of DNA and the presence of rat brain RNA as a primer was sufficient. It differed

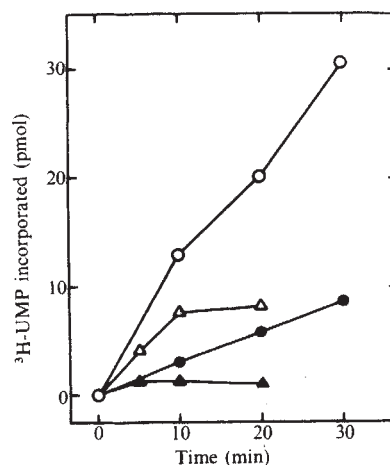


Fig. 2 Time course of RNA synthesis. The reaction mixture and conditions were the same as described in the legend to Table 1. Enzyme and RNA from newborn rat brain: ○, +RNA; ●, –RNA. Enzyme and RNA from adult rat brain: △, +RNA; ▲, –RNA.

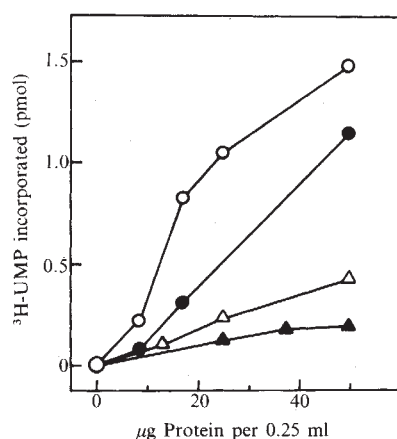


Fig. 3 Dependence of incorporation on concentration of enzyme protein (homopolymer synthesis). Conditions of assay were those described in the legend to Table 4 except for 5 µg of RNA of either adult or newborn brain and 10 minutes incubation. ○, Newborn enzyme with newborn RNA; ●, newborn enzyme with adult RNA; △, adult enzyme with newborn RNA; ▲, adult enzyme with adult RNA.

from newborn enzyme in that the optimum $MgCl_2$ concentration was lower, about 4 to 6 mM. The formation of poly(U) may occur either by addition to poly(U) sequences in the template or by terminal addition to an RNA primer. Poly(U) was much more effective as a primer than poly(A) or poly(C), and poly(G) actually inhibited the residual synthesis occurring in the absence of added primer (Table 4). This means that if

Table 3 Effect of various nucleic acids offered as template for RNA synthesis by adult enzyme

Template (µg per 0.25 ml)	3H -UMP incorporated (pmol)
Experiment 1 Brain RNA	10
poly(A)	5
Poly(U)	10
Poly(G)	5
Poly(C)	10
—RNA	5
Experiment 2 Brain RNA	6
Liver RNA	6
Qβ phage RNA	6
GA phage RNA	6
MS2 phage RNA	6
TMV RNA	6
<i>E. coli</i> tRNA	6
rRNA	6
Calf thymus DNA	7.5
—RNA	6

Conditions of assay were those described in the legend to Table 1 except that various nucleic acids were used as templates. Qβ phage RNA, MS2 phage RNA and GA phage RNA were prepared in our laboratory by the procedure described by Haruna and Spiegelman¹. TMV RNA, tobacco mosaic virus RNA was kindly supplied by Dr Y. Okada (Tokyo University). RNA was isolated from brains (cerebrums and cerebellums) of adults and 1-d-old Wistar rats. Fresh brain was cut up with scissors and stirred for 30 min at 4° C in a solution containing 0.1 M Tris-HCl (pH 7.4) 10 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 5 µg ml⁻¹ DNase and 0.4% (w/v) sodium dodecyl sulphate and incubated at 20° C for 8 min. An equal volume of phenol saturated with cold water was added and shaken vigorously for 15 min at 4° C. After centrifugation at 12,000g for 15 min, the upper aqueous phase was mixed again with an equal volume of water-saturated phenol. Phenol treatment was performed four times and the ether flushed out with nitrogen gas, two volumes of cold (−20° C) ethanol was added and stood overnight at −20° C. The mixture was centrifuged at 9,000g for 10 min, the pellet was dissolved in 5 mM $MgCl_2$, 0.01 M Tris-HCl (pH 7.4). After ethanol precipitation at −20° C, the pellet was again dissolved in 0.01 M Tris-HCl (pH 7.4). The supernatant, after centrifugation at 12,000g for 20 min was used as the template and was stored at −20° C.

Table 4 Characterisation of the reaction of 3H -UMP incorporation by newborn enzyme with UTP as substrate

	Assay mixture (µg per 0.25 ml)	3H -UMP incorporated (pmol)
Experiment 1	Control	7.9
	+ DNase	10
	+ Actinomycin D	2
	+ RNase (+RNA)	10
	(−RNA)	10
	− $MgCl_2$	1.5
	−RNA	2.0
Experiment 2	Control	7.2
	+ ATP	0.2*
	+ GTP	0.2*
	+ CTP	0.2*
Experiment 3	Control −RNA	2.5
	+ RNA	10
	+ Poly(A)	20
	+ Poly(U)	10
	+ Poly(G)	10
	+ Poly(C)	10
Experiment 4	Control	8.3
	−RNA + C.T. DNA	10
Experiment 5	Control (13 mM $(NH_4)_2SO_4$)	7.5
	+ 0.05 M $(NH_4)_2SO_4$	4.5
	+ 0.13 M	1.6
	+ 0.41 M	1.4
	−RNA	1.3

The reaction mixture had a total volume of 0.25 ml and contained 22 µmol of Tris-HCl (pH 7.4), 1.5 µmol of $MgCl_2$, 4.5 nmol of 2-mercaptoethanol, 10 nmol of 3H -UTP (2×10^6 c.p.m. per 10 nmol) and 34 µg of newborn brain enzyme and 10 µg newborn brain RNA. Incubation was for 20 min.

* 0.2 µmol of ATP, GTP or CTP were added to the mixture to test the effect of ribonucleoside triphosphate. Homopolymers were added instead of RNA.

there was terminal addition it had strict primer requirements. When primed by the same primer RNA the activity of the newborn enzyme was several times higher than that of the adult (Fig. 3).

It is not known whether the enzyme fraction contains only one enzyme mediating both types of synthesis or whether there are two or more specialised enzymes. We hope that this point will be clarified by purification of the enzyme fraction. Physiological roles for two enzyme systems are still obscure. It has been reported that homopolyribonucleotide synthesis in embryonic chick retina is involved in selective gene expression¹⁷, and so we assume that our findings that the enzyme activity which is particularly engaged in homopolymeric RNA synthesis in brain tissue is higher in preparations from newborn than adult rats may have physiological significance.

As it is well known that brain RNA changes according to functional activities¹¹, we suggest that the enzyme reaction may participate in brain function, although we have no direct evidence of physiological function of this enzyme in the brain at present. We are now investigating whether this reaction exists in other tissues.

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KATSUHIKO MIKOSHIBA
YASUZO TSUKADA

Department of Physiology,

ICHIRO HARUNA
ITARU WATANABE

Department of Molecular Biology,
Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku,
Tokyo-160, Japan

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No interaction between ultraviolet and X irradiation on chromosome aberrations in cells with trisomy 21

WE have previously shown¹ that ultraviolet irradiation of normal human lymphocytes immediately before or after X irradiation gives an approximately two-fold increase of the frequency of dicentric chromosomes as compared with the yield induced by X rays alone. Ultraviolet light itself induces very few dicentrics. One interpretation of this synergistic reaction might be that lesions induced by X rays are transformed into chromosome breaks by the ultraviolet treatment.

Here we report that no synergistic effect between ultraviolet light and X rays was observed after combined irradiation of blood lymphocytes of individuals with Down's syndrome (trisomy 21). The finding that the chromosome

constitution influences the interaction between ultraviolet and X irradiation is of special interest as it has previously been shown that cells with trisomy 21 have a higher sensitivity for chromosome breakage than normal cells after single irradiation with ⁶⁰Co or X rays^{2,3}.

Unstimulated lymphocytes resting in the G₀ stage were treated with ultraviolet and X rays in three separate experiments. Peripheral blood from three 47, XY, +21 patients and two 46, XX healthy donors was used immediately after venipuncture. The leukocytes were separated as buffy coats, after which the cells were suspended in a phosphate buffer and irradiated in plastic Petri dishes. The irradiations were made at room temperature and the time interval between the ultraviolet (2537Å, 6.1 erg mm⁻² s⁻¹) and X-ray (260 kV, 11 mA, HVL 2 mm Cu, 50 rad min⁻¹) exposure in the combined irradiation was less than 30 s. Conventional lymphocyte cultures were prepared and PHA added. The cultures were incubated at 37°C for 50 h (experiments A and B) and 52 h (experiment C), with colchicine added for the last 2 h. Thus, the sampling of cells was made in the first mitotic division, where the aberration yield should not be affected to any appreciable extent by a mitotic delay⁴. After hypotonic treatment and fixation, air dried preparations were made and stained in Giemsa. The slides were screened for consecutive, well-spread metaphases at 10×10 magnification, and the cells were then analysed for dicentric chromosomes (with accompanying fragments) at high-power magnification.

The present results of the induced yields of dicentric chromosomes are given in Table 1. Normal cells exhibit a synergistic effect between ultraviolet light and X rays in agreement with previous data¹. The 47, +21 cells did not show any significant increase in the dicentric chromosome yield after pre- or post-treatment with ultraviolet as compared with that observed after X-ray exposure alone. The ratio between the number of dicentric chromosomes observed in the combined irradiation to that observed after X irradiation alone is plotted in Fig. 1 for normal cells (including data from ref. 1) and for cells with trisomy 21. The mean values (with standard deviations) of this ratio were

0.23 0.18
 $2.07 \pm \text{---}$ and $1.15 \pm \text{---}$ for normal and trisomy 21 cells,
 0.19 0.14
 respectively.

In the present experiment the X-ray induced dicentric yield is about 50% higher in cells with trisomy 21 as compared with normal cells. The statistical errors are relatively large, however, and a large significant difference

Table 1 Observed number of dicentrics after single and combined irradiations with ultraviolet (UV) light and X rays.

Experiment	Subject chromosome constitution	Treatment	No. of cells analysed	No. of dicentrics	Dicentrics per cell
A	I: 47, XY, + 21	X rays, 100 rad	100	16	0.16 ± 0.04
		UV, 65 erg mm ⁻² + X rays, 100 rad	100	20	0.20 ± 0.05
B	II: 47, XY, + 21	UV, 75 erg mm ⁻²	100	2	0.02
		X rays, 125 rad	150	40	0.27 ± 0.05
		UV, 75 erg mm ⁻² + X rays, 125 rad	150	53	0.35 ± 0.06
	III: 46, XX	X rays, 125 rad	150	45	0.30 ± 0.05
		+ UV, 75 erg mm ⁻²	150	27	0.18 ± 0.04
		X rays, 125 rad	150	68	0.45 ± 0.06
C	IV: 47, XY, + 21	X rays, 150 rad	100	40	0.40 ± 0.07
		UV, 55 erg mm ⁻²	100	41	0.41 ± 0.07
		+ X rays, 150 rad	100	42	0.42 ± 0.07
	V: 46, XX	UV, 75 erg mm ⁻²	100	27	0.27 ± 0.06
		+ X rays, 150 rad	100	47	0.47 ± 0.07
		UV, 55 erg mm ⁻²	100		

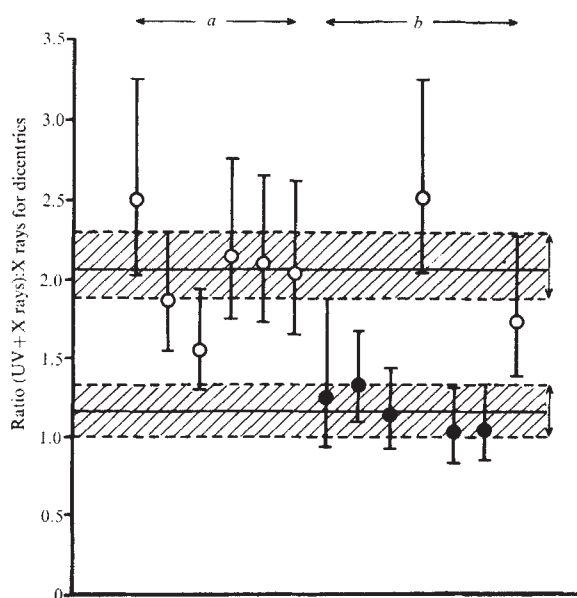


Fig. 1 Ratio between the number of dicentric chromosomes observed in the combined irradiation with that observed after X irradiation alone plotted for male and female normal cells (○) and cells with trisomy 21 (●). *a*, Data from ref. 1; *b*, data from present work.

in radiation sensitivities for the two types of cell is present only in the pooled data of 125 and 150 rad. Sasaki and Tonomura² reported an approximately two-fold increase of the radiation (⁶⁰Co) induced exchange aberration yield in cells with trisomy 21 as compared with normal cells, while the X-ray data by Evans³ give a yield about 40% higher.

The correlation between the higher yield of X-ray induced chromosome exchange aberrations in cells with trisomy 21 in comparison with controls and the lack of a synergistic effect between ultraviolet and X rays in cells with trisomy 21 cannot be unambiguously explained. The synergistic effect observed in normal cells is; however, (with-in experimental error) independent of the ultraviolet exposure in the range 50–100 erg mm⁻². This correlates with the finding by Evans and Norman^{5,6} that the amount of unscheduled DNA synthesis saturates at about 50 erg mm⁻². It is not known whether the ultraviolet dose dependence of unscheduled DNA synthesis is different in cells with trisomy 21.

Blood specimens from the individuals with Down's syndrome were provided by Dr J. A. Hedberg, Björnkulla Institution for the Mentally Retarded, Stockholm.

M. HOLMBERG

Research Institute of National Defense, Department 4,
S-104 01 Stockholm 80 and
Department of Clinical Genetics,
Karolinska Hospital,
S-104 01 Stockholm 60

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Injection of foreign substances into single cells by cell fusion

A GOOD technique for injecting a given substance into an individual cell would be of value in cell biology. We here report studies on a method for injection of foreign substances into cells with erythrocyte ghosts as 'syringes', making use of the cell fusion activity of HVJ. Mammalian erythrocyte ghosts seem to be the most suitable disposable 'syringes', since they have no cytoplasm or nucleus, so contamination with cellular materials at the time of injection is minimal. Among the many mammalian erythrocytes tested, only human and dog erythrocytes showed high fusion capacity under the routine conditions used for treatment with HVJ (ref. 1).

As a preliminary step, we examined whether haemoglobin was injected into a culture cell during its fusion with an intact human red blood cell (RBC). Cells of a cloned line of FL cells originating from a human amnion cell were used as host cells. About 1×10^5 cells suspended in 8 ml of culture medium minimal essential medium (MEM) (with 10% calf serum) were inoculated into a Petri dish, diameter 5 cm, with several round cover glasses, 1 cm in diameter, at the bottom. The dishes were kept for 12 h to allow the cells to settle on the cover glasses. A suspension of 1.5% RBCs was made with balanced salt solution, BSS⁺ (ref. 2) and the suspension was kept at 4° C until use. After removal of the culture medium, the cover glasses on which the FL cells had been plated were washed with cold BSS⁻. To coat the surface of FL cells with HVJ, 1.5 ml of ultraviolet-irradiated HVJ (ref. 3), 1,500 haemagglutinating units⁴, suspended in cold BSS⁻ were added and the culture was kept at 4° C. After 20 min free viruses were washed off with cold BSS⁻. Then, 2 ml of the RBC suspension were poured into the Petri dish and kept at 4° C for 30 min to allow the RBCs to be adsorbed to the surface of HVJ-coated FL cells. Under these conditions, no cell fusion occurred. After completion of the adhesion of RBCs, the BSS was removed carefully and 5 ml of warm BSS⁺ was poured into the Petri dish. The culture was then incubated at 37° C for various periods, during which cell fusion between FL cells and RBCs was expected to occur accompanied by haemoglobin injection into the FL cells. The incubation periods used were 0, 20, 45, 90 min, 1 and 2 d; for incubation for longer than 20 min ordinary culture medium was used. After each of these periods a cover glass was taken out and washed with PBS (phosphate buffered saline, pH 7.2) to remove free RBCs and then stained with benzidine⁵ to detect haemoglobin in the FL cells. The percentages of benzidine positive cells after incubation for 0, 20, 45 and 90 min were 0%, 22%, 34% and 36%, respectively. The injected haemoglobin spread rapidly throughout the cytoplasm. The presence of haemoglobin in FL cells was still clearly demonstrable after incubation for 1 or 2 d. It is significant that all the FL cells into which haemoglobin had been injected looked normal and many dividing cells containing haemoglobin were observable in long-term cultures.

The haemoglobin had to be replaced by other substances without destroying the fusion capacity of the RBCs. For this experiment we chose fluorescein isothiocyanate (FITC) because it is easy to detect. Packed RBCs (1 ml) were haemolysed by addition of 5 ml of cold distilled water. The resulting lysate was centrifuged at 11,000g for 10 min and the supernatant discarded. Six-fold diluted PBS was added to the pellet to remove haemoglobin and the mixture was centrifuged in the same way and the supernatant discarded; this procedure was repeated three times. Six ml of a solution of 0.05 mg ml⁻¹ of FITC in six-fold diluted PBS were then added to the pellet. The mixture was stirred and centrifuged at 11,000g for 10 min and the

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³ Evans, H. J., in *Advances in Physical and Biological Radiation Detectors*, 593–609 (Proceedings of a Symposium, IAEA, Vienna 1971).

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supernatant discarded; this procedure was repeated twice. The pellet was then treated similarly with the same concentration of FITC in half-strength PBS and centrifuged at 11,000g for 10 min. All these procedures were carried out at 4° C. Finally, the pellet was mixed with the same concentration of FITC in 10 ml of full strength PBS and the mixture was incubated at 37° C for 30 min. It was then centrifuged at 7,000g for 10 min and the supernatant discarded. The resulting yellow RBCs were washed with PBS by repeating centrifugation at 1,700g for 15 min until the supernatant became colourless. The external appearance of the yellow RBC containing FITC was indistinguishable from that of intact ones and the FITC fluoresced intensely. For preservation, the cells were suspended in cold PBS with 4 mM ATP and 0.1 mM MgCl₂. It was estimated that each corpuscle contained about 4.4×10^{-3} pg of FITC. No leakage of FITC from the corpuscles was observable during overnight storage.

Cell fusion between FL cells and the yellow RBCs was tested following the procedure described above. In this case, however, on long-term incubation the FL cells died as a result of the cytotoxic effect of the very small quantity of FITC which leaked from the RBCs during reactions with HVJ, so the presence of FITC in FL cells had to be checked after relatively short incubations of 20 min and 60 min. Cover glasses were removed from Petri dishes, washed with PBS, placed on a thin slide with the cells downwards and enclosed in buffered glycerine. Fluorescence was examined in a fluorescent microscope under a dark field and injected FITC in the FL cells was demonstrated. The frequency of injection was less than that of haemoglobin, however, being lower than 1%. Because FITC is highly cytotoxic, considerable numbers of FL cells may have been killed and detached from the surface of the cover glass immediately after the injection started, so the actual rate of FITC injection may in fact have been much higher. This result is encouraging, however, since intracellular injection of FITC was achieved, albeit at low frequency.

There are several possible advantages of this method. First, it is well known that HVJ causes a cell fusion reaction to a greater or lesser extent in almost all cultured cells, so intracellular microinjection into them will be possible. Second, with further improvement of the method it should be possible to control the dose of substance injected into a single cell. Third, it should be emphasised that after injection of haemoglobin FL cells were healthy and continued to divide, so the injection procedure itself is not basically harmful to the cells.

There are also possible difficulties in the method. There are technical limitations to replacing haemoglobin with other substances. During injection of intracellular substances, the RBC membrane is inevitably transplanted into the plasma membrane of the target cell, but it may have no appreciable effect, since during repeated cell divisions molecules of the transplanted RBC membrane will undergo progressive geometric dilution. Moreover, the transplanted RBC membrane may be a good marker to identify cells which have received an injection, because RBC membrane-specific antigens should be detectable by staining labelled antibody⁶.

We plan to examine appropriate conditions for fusion of a single cell with a single RBC.

MITSURU FURUSAWA
TOSHIKAZU NISHIMURA

Laboratory of Embryology,
Department of Biology, Faculty of Science,

MASARU YAMAIZUMI
YOSHIO OKADA

Department of Animal Virology,
Research Institute for Microbial Diseases,
Osaka University, Osaka, Japan

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Mechanisms of sensing chemical gradients by polymorphonuclear leukocytes

ALTHOUGH it has been known for nearly 100 yr that polymorphonuclear leukocytes (PMNs) can show oriented locomotion in response to chemical gradients, that is, they display chemotaxis, the way in which these cells detect and respond to chemical gradients is still not understood¹. These questions are of interest, not only to a study of the inflammatory process, but also to more general problems of oriented cell movements during morphogenesis.

Current studies on bacterial chemotaxis indicate two distinct methods by which an organism can sense a chemical gradient^{2,3}. An organism could sense the concentration of a chemotactic substance at one point, move a certain distance and sense it again, comparing this new level with the previous one. This has been termed a 'temporal' mechanism, as the organism compares between two times. In a 'spatial' mechanism the organism compares the concentration of a chemotactic substance at two or more locations on its body at one time. The mechanisms differ in that the temporal mechanism requires only one receptor somewhere on the organism and a simple memory system, whereas the spatial mechanism requires at least two spatially separated receptors on the organism and implies that the organism is capable of sensing the gradient across its own dimensions^{2,4}. Analysis of films of horse leukocytes initiating and exhibiting chemotaxis demonstrates that these cells utilise the spatial mechanism. This conclusion is supported by Ramsay's observations of a human PMN responding to a moving chemotactic stimulus⁷.

Time lapse films were made of horse blood neutrophilic leukocytes in a thin slide coverslip preparation as they responded to a chemotactic stimulus generated by other leukocytes in contact with a line of aggregated γ globulin that had been previously dried on the slide. The details of the preparation have been described previously⁵.

The magnitude of the chemotactic response was quantitated in three ways. First, the number of cells locomoting toward the chemotactic source was compared with that of cells moving away from it. Of the 80 PMNs filmed, 78 moved towards the chemotactic source, that is, into the 180° sector containing the chemotactic source, no cells moved away and two were immobile. Second, the precision of the orientation of cellular locomotion was determined by comparing the orientation of movement of individual cells with the most direct possible path to the chemotactic source. In this analysis the successive positions of 30 cells were traced by projecting frames of the time lapse films onto drawing paper. As the cells tend to move in straight lines for short periods of time, the traced paths could be broken into segments of straight movement (that is movements having less than a 10° change in direction which were separated by relatively abrupt turns of varying magnitude). The tracing of four such cell paths is shown in Fig. 1. From the distribution of straight segments shown in Fig. 2, it was calculated that 65% of segments were oriented within 30°

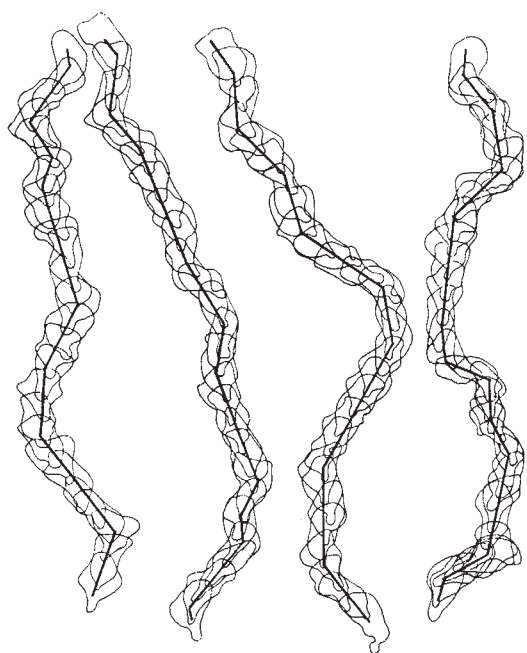


Fig. 1 The tracings of four cell paths is a $16\times$ field immediately adjacent to a line of aggregated γ globulin filmed at $60\text{ frames min}^{-1}$. The film was projected on paper and at every 30th frame the cell images were drawn. The cells move from the bottom of the figure toward the top; the γ globulin line was parallel to the top edge of the figure. Straight lines were drawn through segments of the tracings which showed less than a 10° change in direction. These lines were then analysed for their orientation relative to a perpendicular to the γ globulin line, their length, and the magnitude and direction of the turns linking the various straight segments.

of the most direct path. Third, the degree of chemotaxis was also evaluated with the chemotropism index of McCutcheon by determining the ratio of the length of the most direct path to the chemotactic source to the path length measured from the film tracings⁶. The chemotropism index ranged from 0.61–0.97 with a mean of 0.85 and s.d.m. of 0.01. The index is higher than those previously published^{6–8}. From these observations it can be concluded that PMNs can sense and respond with high accuracy to a chemical gradient. The observed abilities of the PMNs to orient is presumably less than their maximal capability as with a more perfect gradient or a more potent chemotactic substance they could be expected to respond even more accurately.

Studies have shown that bacteria swimming in a gradient of a chemotactic substance have a lower frequency of turning when they are moving up the gradient than when they are moving down^{2,3}. This variation in turning behaviour with the orientation of swimming causes the ratio of cells moving up the gradient over those moving down to exceed one. Previous studies on PMNs have suggested that randomly moving cells have a higher frequency of turns and stops than cells exhibiting chemotaxis^{3,6}. In my present study the frequency and magnitude of turns were examined with PMNs which were moving with various orientations to a chemotactic source. The distance between turns did not alter as a function of the orientation of the locomotion. (coefficient of correlation was -0.169 , $N=68$, $P>0.1$). Thus a cell which deviated 40° from the direct path to the source continued in this direction as far as one headed directly towards the source. In the population studied, no cell was moving at an angle more than 100° from the direct path to the source; in situations of less efficient orientation a significant correlation might exist. In this same population, however, a significant correlation was found

between the magnitude of a turn and the orientation of that cell immediately before the turn (coefficient of correlation $=0.35$, $N=68$, $P\leq 0.01$). The further a cell was oriented from the direct path the greater the magnitude of the next turn tended to be. The correlation, however, was not strong.

Probably an even more important factor in the orientation of moving PMNs than the magnitude of turn is the direction of the turn. Thus 15 out of 16 cases where a cell in the tracings was moving at an angle of 30° or more from the direct path to the chemotactic source, the direction of the next turn by the cell was towards the source; the probability of this bias occurring by chance is less than 0.001.

These behaviour patterns of cells exhibiting chemotaxis are consistent with a spatial mechanism of sensing the gradient. The directional turning response of cells rules out a simple temporal system based on readings at only two points as such a system would not allow the cell to decide in which direction to make its next turn. The data, however, do not eliminate a more complex temporal sensory mechanism which envisions moving cells as having the ability to 'remember' the concentration levels over two preceding intervals as well as the direction of the turn linking the two. This mechanism would give the cell as much information as a spatial mechanism with three or more receptors (knowledge of the concentration at three points in a plane is sufficient to determine the direction and relative steepness of an unidirectional gradient in that plane).

To differentiate between spatial and temporal mechanisms I observed the initial orientation of cells placed in a chemotactic gradient. Only a spatial mechanism should allow cells to detect the direction of a gradient while stationary. The direction of initial locomotion of PMNs placed on a slide bearing a streak of aggregated γ globulin was analysed. Filming was begun 3 min after the preparation was made; at this time the PMNs were still rounded and immobile. Gradually the cells began to extend pseudopods, the cells nearest the line of γ globulin first, followed by cells further away. The direction of the initial locomotion was towards the source of the chemotactic agent. In the microscopic field filmed, 18 out of 20 cells which moved either towards or away from the chemotactic source, extended their first pseudopod and made their initial

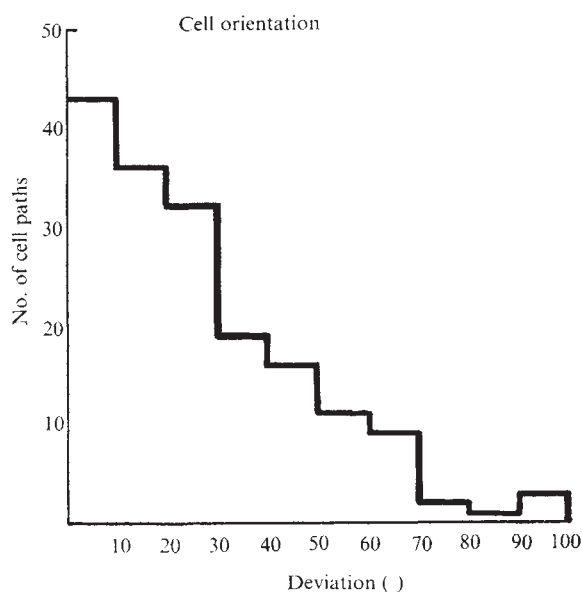


Fig. 2 The number of straight segments of cell paths is plotted against their orientation relative to the perpendicular to the γ globulin line. A total of 172 straight segments taken from the tracings of 30 cell paths were measured.

translocation (of at least $\frac{1}{3}$ their body length) into the 180° sector toward the chemotactic stimulus. This population distribution would occur by chance less than 1 time in 1,000. Thus, the stationary cells are able to sense the gradient across their own dimensions and to respond directionally from the stationary position, an ability characteristic of an organism with a spatial mechanism of detecting gradient (a specialised temporal system where a single receptor is moved over the cells' surface is an alternative, unlikely, possibility).

In response to the chemotactic stimulus the frequency of pseudopod formation increased on the high concentration side of the cell. Before initial migration, the stationary cells did show changes in shape but usually these did not include the formation of structures which could be recognised as pseudopods, either with 16× or 100× microscope objectives. Furthermore, cells greater than 1 cm from the γ globulin line normally remained rounded even after 1.5 h incubation.

How the cell utilises information obtained by the spatial sensory system to selectively form pseudopods is unknown. Possibly there is a time and concentration dependent threshold of stimulation at the membrane which when activated leads to local pseudopod formation and a refractory period inhibiting pseudopod formation on other portions of the cell¹⁰. Alternatively, the cell could have an internal means of comparing the amount of stimulation of its various sides; for instance local stimulation could affect the distribution of some limiting cofactor required for pseudopod formation. Further studies will be required to distinguish between these and other alternatives.

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SALLY H. ZIGMOND*

Strangeways Research Laboratory,
Cambridge, UK

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*Present address: Section of Cell Biology, Yale Medical School, New Haven, Connecticut.

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Optimal recovery of lymphocytes and tissue culture cells following rapid cooling

CELLS die if cooled either too rapidly or too slowly during freezing in the presence of dimethylsulphoxide (DMSO)¹⁻⁴, but survive at an intermediate optimal cooling rate⁵. We have now obtained improved recovery of lymphocytes, lymphoid cell line cells and Chinese hamster tissue culture cells by interrupting rapid cooling with a timed exposure to a single subzero temperature. This method is simple and here we put

forward some theoretical reasons for the improved cell recovery.

Dulbecco's medium containing DMSO (5% v/v) was added to gelatin-separated human peripheral blood lymphocytes immediately before dispensing 0.2 ml aliquots into sterile glass test tubes (10 cm long, 7 mm outer diameter, 5 mm inner diameter). The tubes were immersed either in a constant temperature alcohol bath ($\pm 0.2^\circ\text{C}$, ref. 6) or in a bath of glycerol solution inside a commercial deep freeze. The bath temperature was measured by a calibrated resistance thermometer⁷. After different times at the chosen subzero temperature, the tubes were plunged into liquid nitrogen. A typical sample reached -25°C in less than a minute from room temperature, the subsequent drop to -196°C took less than 20 s. The samples were thawed in a water bath at 37°C for 20 s. The lymphocytes were diluted in one step with 1.0 ml of serum-gelatin and cultured as previously described¹⁻⁵. Recovery was assessed by uptake of ^3H -thymidine (^3H -TdR) on the third or fourth day of culture of lymphocytes stimulated with phytohaemagglutinin (PHA).

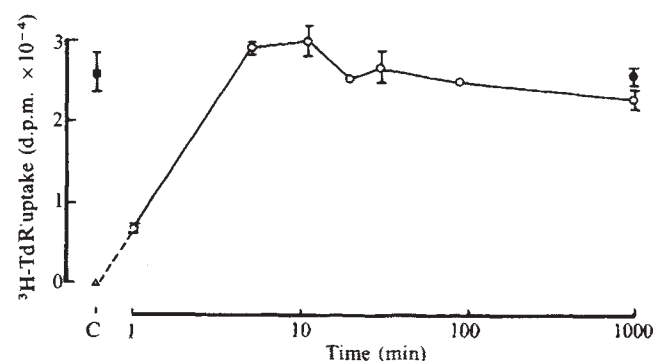


Fig. 1 PHA-stimulated ^3H -thymidine uptake (d.p.m.) of unfrozen control lymphocytes without DMSO (■), and thawed cells frozen in DMSO (5% v/v) either directly to -196°C (Δ) or kept at -25.6°C for different times before either thawing (●) or plunging to -196°C before thawing (○).

Figure 1 shows the recovery of cells held at -25.6°C in the deep freeze before immersion in liquid nitrogen. Complete recovery (100%) of the PHA-induced ^3H -TdR uptake was obtained with cells held from between 5 min and 18 h at the subzero temperature before cooling rapidly to -196°C . There was no recovery of ^3H -TdR incorporation by cells cooled directly to -196°C without exposure at -25.6°C . Using holding temperatures of -22°C , -26°C , -30°C and -40°C the maximum recoveries of PHA-responsive lymphocytes were 18%, 100%, 65% and 40% respectively. Cells held at the sub-optimal temperatures of -22°C and -40°C for 1 to 2 h were damaged on thawing from that temperature; no additional damage was seen when replicate samples were cooled to -196°C before thawing. With DMSO (10% v/v) complete recovery of ^3H -TdR uptake in response to PHA was obtained only after 50 min at -26.3°C in the alcohol bath. It was important to cool the sample rapidly to the subzero holding temperature. Even 0.5 ml samples of cells in polypropylene Sterilin tubes showed a slower recovery with time at -25.2°C in the alcohol bath, in comparison with the 0.2 ml samples in the glass test tubes.

Recovery was also observed with two other cell types known to succumb to rapid freezing. Lymphoid cell line cells (Cla 4) showed 100% recovery after 30 min at -26°C before cooling to -196°C . Recovery was assessed by ^3H -TdR uptake for 24 h. Chinese hamster lung fibroblast cells (V79-379A) were grown and collected as previously described⁸. Duplicate samples were frozen in the same manner as the lymphocytes in Eagle's minimal essential medium with DMSO (5% v/v). On thawing, the samples were plated for colony assay. Cells frozen directly to -196°C from room temperature showed no colony growth on thawing (Fig. 2). Between 1 min and 10 min at -26.0°C

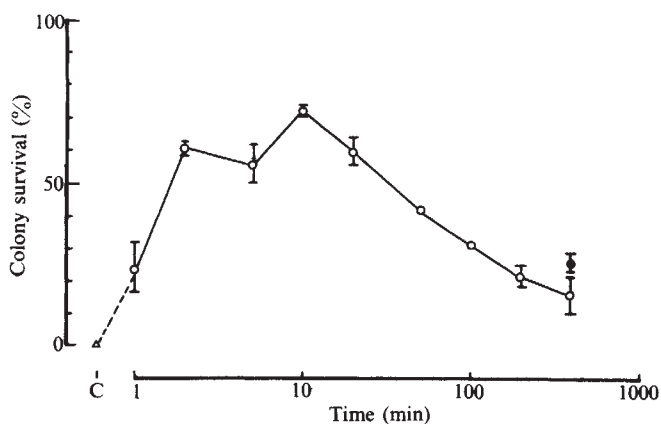


Fig. 2 Colony growth (as % of unfrozen control) of Chinese hamster tissue culture cells thawed after freezing in DMSO (5% v/v) either directly to -196°C (Δ) or kept at -26.0°C for different times before either thawing ($\bullet$) or plunging to -196°C before thawing ($\circ$). The plating efficiency of the unfrozen control was 66.5%.

the survival of tissue culture cells thawed from liquid nitrogen increased to a maximum of 73% at 10 min. With longer times the survival decreased.

These results demonstrate a simple method of obtaining a high survival of different cell types after storage in liquid nitrogen. With human lymphocytes we obtained complete survival of responses to PHA with a low concentration of DMSO (5% v/v). Using conventional optimal cooling rate techniques, this concentration does not allow complete survival⁹.

The basis of the present method is the realisation that there is a zone of subzero temperature, exposure to which protects cells against damage due to the subsequent stresses of both rapid cooling to -196°C , and rapid thawing and dilution. This was shown some time ago by Luyet and Keane¹⁰ for bull spermatozoa and by Taylor¹¹ for chick skin cells. Our results show that the protective properties of different subzero temperatures are affected by physical factors such as the initial concentration of DMSO before freezing. In addition, different cell types respond differently to the same subzero protective environment, thus providing a possible method for their separation.

We propose that this temperature zone conferring protection to cells against subsequent freezing and thawing damage explains the established finding that most cells will survive freezing if cooled slowly (for example at $2^{\circ}\text{C min}^{-1}$ for lymphocytes in DMSO 5% v/v)^{1,5} rather than rapidly. The disadvantage of the conventional optimal cooling rate appears to be two-fold. First, damage may be produced by the initial slow attainment of the best protective temperature (for example the cooling from 0°C to -26°C for lymphocytes in DMSO 5% v/v); second, if slow cooling is continued below the protective zone damage may be caused. The beneficial effects of the two stage cooling rates sometimes used^{12,13} can thus be explained. The logical improvement is rapid cooling to the optimal protective temperature, a period at that temperature to confer protection, followed by a rapid cooling to storage temperatures.

We have shown that two separate processes determine the survival of the cells thawed from -196°C ; these are damage at the holding temperature and protection against damage on subsequent freezing and thawing.

The mechanism of the protection with time is unknown. It bears one similarity to the thermal shock system¹⁴ where red cells cooled slowly from -37°C to 0°C in hypertonic sodium chloride are haemolysed less than cells cooled rapidly^{15,16}. The rapidly cooled red cells could be protected, however, by increasing the time of exposure to the hypertonic conditions before cooling¹⁶. The mechanism of protection at the subzero temperature may involve membrane changes

induced by the hypertonic conditions, water or solute movements across the cell membrane or the redistribution of ice.

We believe that these results will form the basis for simple improved techniques for cellular preservation.

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JOHN FARRANT
STELLA C. KNIGHT
L. E. MCGANN
JACQUELINE O'BRIEN

*Divisions of Cryobiology and Surgical Sciences,
Clinical Research Centre,
Harrow, Middlesex*

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Are there differences in contact inhibition of movement between normal and malignant cells

STOKER¹ claimed that the malignant cells transformed from BHK cells by polyoma virus showed a lack of contact inhibition of movement unlike their progenitor strain BHK 21 C 13. One of us² reported that measurements made of the degree of contact inhibition shown by these two cell types showed that both displayed the same degree of contact inhibition. Measurements were, however, made at one culture age and population density of cells. It is known that contact inhibition of movement of embryonic chick fibroblasts varies with both population density and culture age³. Neither Stoker nor Curtis examined the effects of these factors on the contact inhibition of Py or C13 cells. Thus the disagreement between the results of Stoker and one of us is ripe for further investigation. We report here measurements on the contact inhibition of these two cell types at different culture ages and densities.

BHK C13 and Py cells cultured in this department and originally derived from the Department of Virology, University of Glasgow were obtained from stock cultures by trypsinisation. The cells were grown in Eagles +10% calf serum 10% tryptose phosphate medium. Cultures were started with inoculum densities of 6.4×10^5 , 3.2×10^5 and 1.5×10^5 (C13) 9.0×10^5 , 4.5×10^5 and 1.5×10^5 (Py) cells per dish (60 mm Polystyrene) in order to obtain different population densities at any one culture age. Cultures were fixed at 8, 20, 24 and 48 h after inoculation. They were stained with Harris's haematoxylin and contact inhibition measured by

Table 1 Variation of mean overlap ratio with culture age and population density

a Age in culture (h)	x_1		8	20	24	48			
		C13	0.183	0.219	0.211	0.298			
b Population density	x_2	Py	0.357	0.262	0.255	0.284			
		< 70		70-74.9	75-79.9	80-84.9	85+		
		C13	0.207	0.219	0.259	0.212	0.251		
		Py	0.456	0.301	0.302	0.294	0.265		
c Statistical analysis		Σx_1^2	Σx_2^2	$\Sigma x_1 y$	$\Sigma x_2 y$	Σx_1^2	Σx_2^2	Σy^2	n
	C13	5,061	2,875	14.6	9.90	2,159	0.080	23	
	Py	2,611	532	-5.4	-3.74	2,665	0.059	20	
	Pooled	7,672	3,407	9.2	6.16	4,824	0.139	43	
	Regression coefficient			Deviations from regression					
	b_1	b_2			Sum of squares		Mean square		
	0.0021	0.0019		C13	0.0316		0.0014		
	-0.0012	-0.0008		Py	0.0496		0.0027		
				Sum	0.0812		0.0041		
				Pooled	0.0258		0.0006		
							$F = 13.32$ (d.f.1:40)		

* Expressed as cells per counting area ($1.8 \times 10^{-8} \text{ m}^2$).

† y is overlap index.

counting the number of overlaps in a known area of measured cell population. Overlap index¹ was used as a measure of contact inhibition.

The results are tabulated in reduced form in Table 1a and b. The full results were analysed by multiple covariance analysis in Table 1c. The planes of the regression equations for C13 and Py cells are significantly different, $F=13.32$, d.f.1:40 $P<0.5\%$. Comparing the differences in slope of the regression coefficients for culture age (x_1) alone and for population density (x_2) alone respectively we find that both pairs of coefficients are significantly different. ($F=10.29$ $P<0.5\%$ for x_1 , $F=36.8$, $P<0.5\%$ for x_2).

Thus the response of Py and C13 cells in their overlap indices to culture age and population density differ. Overlap index increases with population density and age in the normal C13 cells, in other words contact inhibition of movement decreases with these changes. But Py cells show the inverse situation. Contact inhibition of Py cells increases with culture age and population density. In consequence it is possible to choose culture ages and population densities such that cultures of both cell types will show the same degree of contact inhibition of movement; presumably this is what happened in earlier work on these cells. But the results also show that Py cells never show a lack of contact inhibition. It will be important to discover whether similar situations exist in other comparisons that have been made in the contact inhibition of normal and malignant cells.

At the moment we can only speculate about the cause of this difference. It is well known that there are surface and other changes in cells as culture age and population density increase⁵⁻⁷ though the effects of age and density do not seem to have been separated in such work. Possibly one or more of these changes is related to the changes in contact inhibition we have observed. In many of these situations malignant and normal cells differ in their reaction to culture age and population density.

MARLYN TURBITT*
A. S. G. CURTIS

Department of Cell Biology,
University of Glasgow,
Glasgow G12 8QQ, UK

*Present Address: Department of Pathology, Glasgow Royal Infirmary.

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New fungal viruses capable of reproducing in bacteria

MANY authors have reported the isolation from fungi of virus particles containing double-stranded RNA¹⁻⁵. It has also been found that extracts of *Penicillium brevi compactum*, *P. chrysogenum* and *P. stoloniferum* can lyse certain bacteria. Fractionation of such extracts and also disruption of spores have revealed the presence of several types of virus particles differing in their biochemical and biological properties. This series of viruses was called PBV, with reference to the host (P) and to their ability to lyse bacteria (B).

Table 1 shows the results of titration of an extract of the mycelium, supernatant and spores of the three species of fungi mentioned above with the cells of *Escherichia coli* C and *E. coli* SK. The infectivity towards bacteria increases as the mycelium grows. No free virus was detected in the supernatant of *P. brevi compactum* after the mycelium was removed, whereas with the other two fungi virus was found in both the mycelium and the culture medium. Viruses which could multiply in bacteria were also found in preparations of the disrupted spores, as well as in preparations of disrupted mycelium not subjected to ammonium sulphate fractionation. In the latter case, the titre of virus in suspension was 1.5-2.0 log lower.

When the fungi were grown in the presence of 250 $\mu\text{g ml}^{-1}$ neomycin, which has a wide spectrum of antibacterial action, the quantity of PBV determined by titration with bacteria does not change as compared with the control without antibiotic. No bacteria were detected at any time in the fungal culture. The results were the same with different strains of *P. brevi compactum* (13 strains have been studied). Figure 1 shows the results of equilibrium centrifugation of mycelial extracts in a Cs_2SO_4 density gradient. The gradient shows several peaks of infectivity to bacteria. The positions of these peaks are different for *P. brevi compactum* and *P. stoloniferum*.

A further investigation has shown that in *P. brevi compactum* extracts there are two types of viruses infective to bacteria (PBV1 and PBV2) in the regions of density 1.38 and 1.40 g cm^{-3} . In the 1.32 g cm^{-3} region⁶, a double-stranded RNA-containing mycovirus is present, which seems to be somewhat smaller in comparison with the mycovirus of

P. brevis compactum described by Wood *et al.*⁷ (300 Å in diameter as measured by us compared with 400 Å indicated by Wood). In the 1.42 g cm⁻³ region there is a third virus, PBV3. The 'light' zones of the density gradient (1.25–1.32 g cm⁻³) also show some infectivity with respect to bacteria.

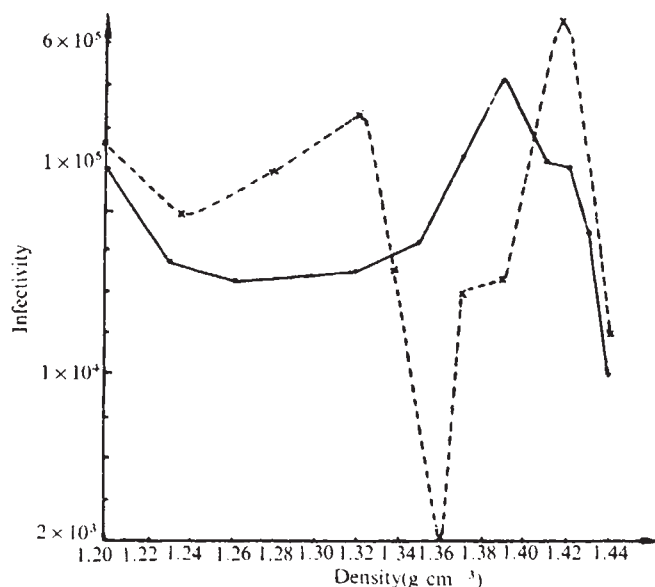


Fig. 1 Equilibrium centrifugation of the extract of *P. brevis compactum* and *P. stoloniferum* mycelium in Cs₂SO₄ density gradient. Preparation and concentration of the extract with ammonium sulphate were carried out as indicated in the legend to Table 1 (the extract of *P. brevis compactum* mycelium was concentrated 10-fold, the extract of *P. stoloniferum*, 1,000-fold). The extracts were layered on the preformed Cs₂SO₄ gradient (1.22–1.44 g cm⁻³) and centrifuged for 16 h in a 3 × 5 ml rotor at 35,000 r.p.m. in a Superspeed 50 centrifuge. In the gradient fraction (0.27 ml) infection titre was determined with *E. coli* SK cells by Gracia⁸ without dialysis. Under the dilution used the Cs₂SO₄ concentration was low and did not influence the results of titration. —, *P. stoloniferum* extract; ---, *P. brevis compactum* extract.

Pure lines of PBV1, PBV2 and PBV3 were obtained (some of their properties are presented in Table 2).

PBV1 and PBV3 comprise icosahedral particles with long noncontractile tails, resembling λ phage; PBV2 is an icosahedral particle with a short tail, resembling T5 phage⁶. The nucleic acid of viruses PBV1 and PBV2, judging by the colour reaction, *T_m* and buoyant density is a typical double-stranded DNA. PBV1 has an extremely wide spectrum of antibacterial action; it can lyse *E. coli* B, *E. coli* C, *E. coli* K12 and *E. coli* SK; PBV2 lyses only *E. coli* SK. PBV3 was infective towards *E. coli* C, *E. coli* B and *E. coli* SK, and has an abnormally high buoyant density in Cs₂SO₄.

Many facts lead us to believe that titration of mycelium extracts on bacteria did reveal the fungal viruses and not some fungal substances which could induce lysogenic prophages in the bacterial cells. Some of these data are summarised in Table 3. First, definite mycelium fractions after equilibrium centrifugation gave several types of colonies on the same bacteria, differing in size and morphology. From these colonies pure viral lines were isolated which after many passages through bacteria had the same buoyant density and gave the same type of colonies as respective viruses in the fungal extracts. This is evident particularly in the case of PBV3 which has an abnormal buoyant density in Cs₂SO₄.

Second, antisera against three isolated and purified PB viruses grown in bacteria neutralise the infectivity of the fungal fraction towards bacteria. The fraction of density 1.42 g cm⁻³ contains chiefly PBV3 (Table 3) which gives large clear colonies with a halo on *E. coli* SK. PBV1 and PBV2 give large clear colonies without haloes and large turbid colonies respectively and represent an admixture of PBV3 as a result of overlapping zones occupied by these viruses. The same is true for the fractions of density 1.40 and 1.38 g cm⁻³. Neutralisation experiments with other fractions (1.32 and 1.38 g cm⁻³) are now under way.

Thus, we conclude that fungal extracts contain virus particles infective towards bacteria. Otherwise *E. coli* C or *E. coli* SK cells would have to contain at least four different prophages which can be induced by fungal substances and at the same time do not restrict the subsequent infection by

Table 1 Titration of Mycelium preparations on *E. coli* C and *E. coli* SK

Fungus species	Time of growth (h)	Infectivity per g biomass		Infectivity titre in culture medium	
		<i>E. coli</i> C	<i>E. coli</i> SK	<i>E. coli</i> C	<i>E. coli</i> SK
<i>P. brevis compactum</i> N 192	6	1 × 10 ²	3 × 10 ²	0	0
	17	8.5 × 10 ²	2.6 × 10 ³	0	0
	24	1.2 × 10 ³	4 × 10 ³	0	0
	72	1 × 10 ⁵	2 × 10 ⁵	0	0
	96	2 × 10 ⁶	9 × 10 ⁶	0	0
	Intact spores	0	0	0	0
<i>P. stoloniferum</i> 1458	Disrupted spores	1 × 10 ³	5 × 10 ³	0	0
	72	2 × 10 ³	—	9 × 10 ²	—
<i>P. chrysogenum</i> 194	48	9 × 10 ²	—	7 × 10 ³	—

The sterile growth conditions of the fungus have been described previously⁶. Twice each day the fungus was seeded to detect bacterial flora. The mycelium was separated from the culture medium by filtering through paper filters and then disintegrated either by grinding with quartz powder or by ultrasonic treatment. Many PB viruses are extremely sensitive to temperature, so it must be kept as low as possible during treatment (0.4° C). The disrupted mycelium was extracted with 0.1 M NaCl in 0.05 M phosphate buffer pH 7; the virus was then either concentrated by precipitating with 30% ammonium sulphate or directly titrated on bacteria, using the method of Gracia⁸. The spores were treated in a similar way. All the data in the table refer to the culture medium and mycelium extracted per g crude mycelium.

Table 2 Some characteristics of viruses of the PBV series (from *P. brevis compactum*) and their DNAs

Virus species	Buoyant density		Size of head Å*	<i>T_m</i> of DNA		Buoyant density	
	in Cs ₂ SO ₄ g cm ⁻³	in CsCl g cm ⁻³		1 SSC	0.1 SSC	in CsCl	in Cs ₂ SO ₄
PBV1	1.38	1.49	425 ± 25	88.5°C	72.8°C	1.7078	1.42
PBV2	1.40	1.48	525 ± 25	87°C	72°C	1.7035	1.42
PBV3	1.42	1.51	—	—	—	—	—

The PB viruses were grown according to the standard procedure⁶ in a synthetic medium to which broth was added and were then purified by chromatography on DEAE-cellulose, differential centrifugation and equilibrium centrifugation in a Cs₂SO₄ density gradient. DNA was isolated by the phenol method. The DNA preparations were dissolved in 1 SSC. Standard procedures were used for melting of DNA and for reactions with orcinic acid and diphenylamine. The buoyant density of DNA was determined in an analytical centrifuge with known markers (DNA from phage Sd or T5).

*The sizes of the particles are those reported in our preliminary communication⁶.

Table 3 Comparison of some properties of PBV originating from fungi and bacteria

Source of Virus*	Buoyant density in Cs ₂ SO ₄ (g cm ⁻³)	Colonies on bacteria	
		Type	Diameter (mm)
Extract of <i>P. brevis compactum</i>	1.42	Clear with halo ‡	1.5–2.0
		Clear without halo	1.5–2.0
		Turbid	0.6–0.8
The same + antiserum against PBV2	1.42	Clear with halo ‡	1.5–2.0
		Clear without halo	1.5–2.0
The same + antisera against PBV1 + PBV3 †	1.42	Turbid	0.6–0.8
PBV3 (<i>E. coli</i> C)	1.42	Clear with halo	1.5–2.0
Extract of <i>P. brevis compactum</i>	1.38	Turbid ‡	0.6–0.8
PBV2 (<i>E. coli</i> SK)	1.38	Clear without halo	1.5–2.0
		Turbid	0.6–0.8
Extract of <i>P. brevis compactum</i>	1.40	Clear without halo ‡	1.5–2.0
		Turbid	0.6–0.8
PBV1 (<i>E. coli</i> C)	1.40	Clear with halo	1.5–2.0
		Clear without halo	1.5–2.0
Extract of <i>P. brevis compactum</i>	1.32	Different types, clear and turbid	0.5–1.5
Extract of <i>P. stoloniferum</i>	1.40	Clear	1.5
Extract of <i>P. chrysogenum</i>	—	Turbid	1.0–1.3

*Fungal extracts were obtained and centrifuged in Cs₂SO₄ gradient, as indicated in the legend to Fig. 1. The fractions with indicated buoyant density were titrated on *E. coli* SK. The pure lines of PB viruses were grown on *E. coli* C (PBV1, PBV3) or *E. coli* SK (PBV2) purified as described in Table 2 and titrated under the same conditions as fungal extracts (see Table 1 and Fig. 1).

† The antisera against PB viruses were obtained by usual method of immunisation of rabbits with purified PBV preparations (titre 5×10^{10} plaques ml⁻¹)¹⁰. The results indicated in the table were obtained with antiserum dilution 1:10, which provided the 100% neutralisation, antisera diluted 1:100 neutralised the infectivity in fungal extracts due to PBV2 (turbid colonies) and PBV1 + PBV3 (clear colonies) for 88% and 90% respectively. Normal rabbit antisera did not influence the PBV infectivity in fungal extracts.

‡ The predominant type of colonies.

infective virus originated from this prophage.

Like the double-stranded RNA mycoviruses, the viruses of the PBV series may be isolated only from the cultures of *P. brevis compactum*, and *P. stoloniferum* with modified morphology and from the strains of *P. chrysogenum*, which produce penicillin. The 'healthy' cultures of these fungi or cultures not producing the antibiotic produce neither mycoviruses nor PB viruses. Attempts to infect 'healthy' cultures with PBVs have so far failed.

In summary, mycelium of *P. brevis compactum*, *P. chrysogenum* and *P. stoloniferum* produces, in addition to known mycoviruses, DNA-containing viruses capable of reproducing in bacteria. The relationship of these viruses to the host fungus is still obscure; it is possible that PBVs have a temperate lysogenic relationship with the fungus.

T. I. TIKCHONENKO
G. A. VELIKODVORSKAYA
A. F. BOBKOVA
YU. E. BARTOSHEVICH
E. P. LEBED
N. M. CHAPLYGINA
T. S. MAKSIMOVA

The D. I. Ivanovsky Institute of Virology of the
USSR Academy of Medical Sciences,
Institute of New Antibiotics of the USSR Academy of
Medical Sciences, Moscow, USSR

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Estimation of the size of a T-cell-induced lytic lesion

The specific cytolytic activity exhibited *in vitro* by thymus-derived (T) lymphocyte populations from immune animals (see review in ref. 1), is germane to allograft rejection^{2,3} and to pathways by which neoplastic tissue can be destroyed *in vivo*^{4,5}. The mechanism involved, however, remains controversial (for one viewpoint see ref. 6) and the nature of the lytic lesion is unknown.

T-cell-mediated lysis seems to be unrelated to the complement system, for not only can lysis take place in the absence of serum, but added complement augments neither the rate nor the extent of lysis⁷. Perhaps more pertinently, antisera against complement components C₂, C₃ and C₅ do not interfere with the lytic process⁸.

Time-lapse cinematography of lymphocyte-mediated cytotoxicity revealed lysis as a ballooning phenomenon, in which the antigen-bearing (target) cell became swollen and ruptured after contact with a lymphocyte⁹. Studies from this laboratory on the efflux of material of different molecular sizes from injured target cells have established that the T-cell-induced lytic lesion is progressive¹⁰. Low molecular weight markers (ATP, ⁸⁶Rb) were shown to efflux from the damaged target cell before macromolecules (⁵¹Cr-protein, ³H-DNA) could pass through the cell membrane. These findings suggest that the original dimensions of the lytic lesion are smaller than the effective diffusion radius of the ⁵¹Cr-protein complexes, and are incompatible with 'big-bang' concepts of lymphocyte-induced target cell lysis.

The preferential early efflux of low molecular weight material from target cells suggested that the eventual rupture of the membrane, and the swelling of the target cell observed morphologically, were due to colloid osmotic swelling. This is supported by the findings reported here, for by means of techniques^{11, 12} used earlier to characterise complement lesions, it was found that when cultures of lymphocytes and target cells were maintained in a solution of high molecular weight (>40,000) dextran, ⁵¹Cr-protein release from the target cells could be inhibited while ⁸⁶Rb efflux continued. Using a series of dextrans of varying molecular size, it was inferred that the initial T-cell-induced lytic lesion in a mastocytoma target cell is of the order of 35–45 Å in radius.

The assay system used, the lysis of DBA/2 mastocytoma cells by spleen cells from alloimmunised C57BL/6 mice, has been described in detail (see legend to Fig. 1)^{13, 14}. The same system was used to measure specific ⁵¹Cr and ⁸⁶Rb efflux¹⁰. During the assay times used, specific release of both labels was linear after a lag of 10–15 min for ⁸⁶Rb and approximately 45 min for ⁵¹Cr. The assays were of comparable sensitivity, in that the minimal number of lymphocytes required to cause significant release of label was equal¹⁰. Typical values for isotope release from 10⁵ target cells with 10⁷ spleen cells is shown in Table 1.

We have shown¹⁰ that target cell damage after addition of lymphocytes occurs within 15 min, and is characterised by efflux of low molecular weight material. Macromolecules are only released later (>45 min). If the efflux of high molecular weight material results from osmoregulatory damage, caused by water influx through the initial lesions, then maintaining

Table 1 Release of ^{51}Cr and ^{86}Rb from DBA/2 mastocytoma cells in the presence of immune C57BL/6 spleen cells

Time after target cell addition (h)	% ^{51}Cr release		% specific cytolysis	^{86}Rb release			
	Immune	Normal		Time (min)	Normal (c.p.m.)	Immune (c.p.m.)	Specific ^{86}Rb release (Δ c.p.m.)
1	9.2 $\pm$ 1.8	3.6 $\pm$ 0.4	5.6	15	541 $\pm$ 17	625 $\pm$ 20	84
2	22.9 $\pm$ 4.0	4.1 $\pm$ 0.2	18.8	30	935 $\pm$ 22	1,360 $\pm$ 18	425
3	40.4 $\pm$ 3.7	5.2 $\pm$ 0.3	35.2	45	1,294 $\pm$ 8	2,124 $\pm$ 75	830
4	56.3 $\pm$ 5.2	6.5 $\pm$ 0.5	49.8	60	1,669 $\pm$ 13	2,871 $\pm$ 12	1,202

C57BL/6 spleen cells from unimmunised (normal) animals or from mice immunised 10 d previously with 10^7 DBA/2 mastocytoma cells intraperitoneally were incubated with 10^5 ^{51}Cr DBA/2 mastocytoma cells for periods up to 4 h (or with 10^5 ^{86}Rb target cells for periods up to 1 h). For the ^{51}Cr release percentage isotope release was calculated relative to the total number of cell-associated counts added to the system. Percentage specific cytolysis merely as the arithmetic difference between release in the presence of immune cells and that with normal cells. Values given are means $\pm$ s.d. of quadruplicate assays in one experiment. For the ^{86}Rb assay, because of high turnover of ^{86}Rb in lytic assays and the variability in reporting specific cytolysis from various laboratories (contrast for example the calculation above with that reported in ref. 13), it is considered preferable here to report raw data. The total cell-associated counts at time zero were $4,646 \pm 420$ c.p.m., the total supernatant counts in the presence of normal and immune lymphocytes is given as c.p.m. $\pm$ s.d. of quadruplicate assays. The difference between the mean values was taken as specific ^{86}Rb release. Percentage inhibition of cytolysis in this system was calculated relative to this value.

the target cells in solutions of high molecular weight (non-penetrating) molecules should prevent the progression of the lesion, and prevent macromolecular efflux^{11,12}.

Using dextrans of various molecular weights, we established that solutions inhibiting cytolysis (^{51}Cr release) were of high intrinsic viscosity. It was considered important, therefore, to establish that inhibition of ^{51}Cr release was due to prevention of macromolecular release from cells bearing a lytic lesion, rather than to inhibition of lymphocyte-target cell interactions by solutions of high viscosity^{9,15}. We argued that if inhibition of both ^{86}Rb and ^{51}Cr release was observed, this represented an inhibition of lymphocyte-target cell interaction. If, on the other hand, ^{86}Rb efflux was demonstrable when ^{51}Cr was not, this was regarded as inhibition of the progression of the lesion. Such arguments depend on the following suppositions: (i) that ^{86}Rb and ^{51}Cr are released from injured cells by basically the same mechanism; and (ii) that ^{86}Rb and ^{51}Cr are located either in the same intracellular pool, or that they have equal access to holes in the cell wall.

It follows that inhibitors of cell interactions should affect ^{86}Rb and ^{51}Cr release comparably. The effect of cytochalasin B, an inhibitor of cytolysis¹⁶⁻¹⁹, was thus assayed. Cytochalasin B ($0.4\text{--}6\text{ }\mu\text{g ml}^{-1}$) similarly inhibited ^{86}Rb efflux and ^{51}Cr release (Table 2). Additionally, we observed¹⁰ that an inhibitor of the potassium pump (ouabain) affected neither ^{86}Rb nor ^{51}Cr release. These findings are both consistent with the suppositions made above.

Parallel inhibition of ^{86}Rb and ^{51}Cr release was shown when lymphocyte-target cell interactions were made in a dextran solution of mean molecular weight 9,300 (Pharmacia size T10). At concentrations of 20 mM, complete inhibition of specific ^{86}Rb and ^{51}Cr release was found. Decreasing dextran concentrations resulted in a dose-dependent and parallel

Table 2 Inhibition of specific ^{86}Rb and ^{51}Cr efflux in the presence of cytochalasin B

Concentration cytochalasin B ($\mu\text{g ml}^{-1}$)	% Inhibition specific cytolysis	
	^{51}Cr	^{86}Rb
0.375	10.0	14.2
0.75	35.2	39.8
1.5	52.0	47.6
3	85.1	79.2
6	97.2	93.4

10^7 immune C57BL/6 lymphocytes (10 d after 10^7 DBA/2 mastocytoma cells intraperitoneally) were incubated with 10^5 ^{51}Cr DBA/2 mastocytoma cells for 4 h at 37°C (or with 10^5 ^{86}Rb target cells for 60 min) in the presence of cytochalasin B. The percentage inhibition of specific cytolysis was calculated relative to control tubes without inhibitor. Data represents mean values of two experiments in each of which the assays were performed in duplicate. Replicate assays in each experiment varied $<5\%$; values from one experiment to the other varied approximately 10% . Cytochalasin B had no effect on the release of either isotope in control cultures.

inhibition of specific ^{86}Rb and ^{51}Cr release (Fig. 1a).

Dextrans of mean molecular weight 70,000 (Pharmacia T70) and 231,000 (Pharmacia T250) inhibited specific ^{51}Cr release significantly more than they did ^{86}Rb flux (Figs. 1b and c). For example, in 0.5 mM, 70,000 molecular weight dextran, specific ^{86}Rb release was suppressed only 6.1% (range 4.2–8.1), whereas ^{51}Cr release was inhibited 38.0% (range 35.0–43.2). Similarly, in 0.1 mM, 231,000 molecular weight dextran, ^{86}Rb release was inhibited 6.5% (range 4.3–9.5) whereas

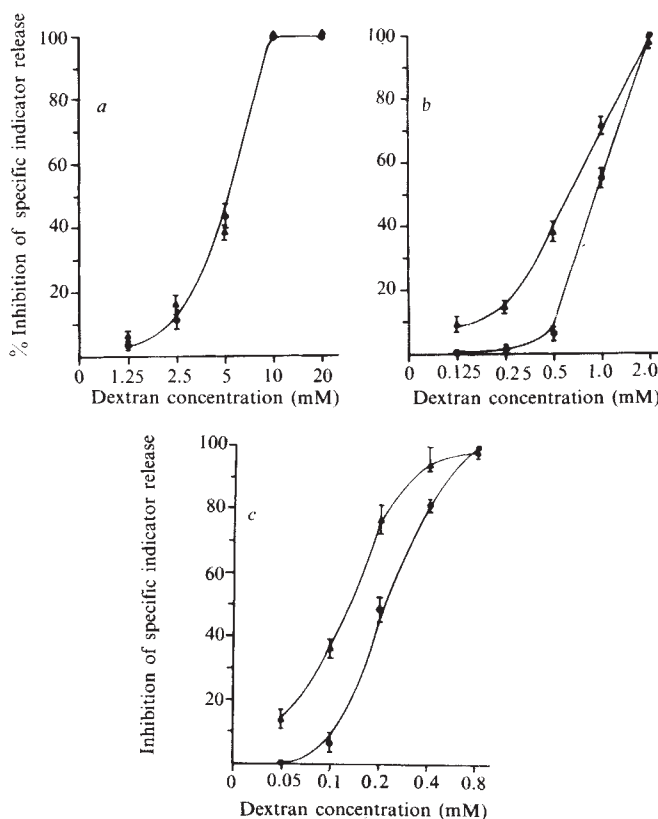


Fig. 1 C57BL/6 spleen cells (10^7) derived from mice immunised 10 d earlier with 10^7 DBA/2 mastocytoma cells intraperitoneally, were incubated with 10^5 mastocytoma cells labelled either with ^{51}Cr (Δ) or with ^{86}Rb ($\bullet$). The cells were mixed in Eagle's minimal essential medium containing 10% foetal calf serum and varying concentrations of dextran. Cytolysis was evaluated 1 h (for ^{86}Rb assays) or 4 h (for ^{51}Cr assays) later. Specific cytolysis was calculated after subtraction of isotope release from control cultures containing 10^7 normal C57BL/6 spleen cells in the same concentrations of dextran. Inhibition of specific cytolysis was calculated relative to cultures containing no dextran. The values given are means and range of quadruplicate cultures. Molecular weight: (a) 9,300; (b) 70,000; (c) 231,000.

Table 3 Addition of cytochalasin B or dextran solutions to cytolytic reactions; effect on subsequent ^{51}Cr release

Reacting cells	Inhibitor added after 1 h of incubation†	% Specific ^{51}Cr release in the 2 h following inhibitor addition		
		Expt 1	Expt 2	Expt 3
10 ⁷ immune C57BL/6 spleen cells	None (medium alone)	20.5 (5.2*)	32.3 (3.1*)	28.7 (6.4*)
	Cytochalasin B	7.3	11.5	8.9
	Dextran 9,300	5.8	—	7.5
	Dextran 70,000	0.2	0.1	0.7
	Dextran 231,000	—	0.5	0.2
10 ⁵ ^{51}Cr DBA/2 mastocytoma cells	BSA	—	3.1	—

* Percentage specific lysis at time of inhibitor addition.

† Inhibitors were added to give a final concentration known to be sufficient to totally inhibit specific ^{51}Cr release if present at time of lymphocyte addition to target cells. These final concentrations were: cytochalasin B, 6 $\mu\text{g ml}^{-1}$; dextran 9,300, 20 mM; dextran 70,000, 2 mM; dextran 231,000, 0.8 mM; BSA, 25 g%. Values given are means of triplicate assays. Replicates in all cases varied < 10%.

Neither dextran nor cytochalasin B solutions affected spontaneous ^{51}Cr release in parallel cultures containing normal lymphocytes.

^{51}Cr release was inhibited 36.2% (range 33.0–39.1). These findings suggest that the high molecular weight dextrans prevent hole enlargement.

We have previously shown that when cytochalasin B is added to a lytic reaction in progress, ^{51}Cr release continues for a period of approximately 1 h before complete inhibition is demonstrable¹⁷. It seems reasonable to assume that such release is derived from target cells which bore holes before addition of inhibitor, but that such lesions were not initially large enough to accommodate macromolecular passage. It follows that hole enlargement continued in the presence of cytochalasin B and that inhibition was due to prevention of new lymphocyte–target cell interactions¹⁸.

When low (9,300) molecular weight dextran was added to a lytic reaction already in progress, ^{51}Cr release continued in a manner comparable with that found with cytochalasin B (Table 3). In contrast, addition of 70,000 and 231,000 molecular weight dextrans completely inhibited subsequent ^{51}Cr release. Presumably only those molecules which are too large to penetrate the initial hole can afford osmotic protection against water influx (and the subsequent ^{51}Cr efflux).

Dextran solutions of 40,000 molecular weight also gave protection against ^{51}Cr macromolecular efflux, although less than that obtained with 70,000 molecular weight material. Minimal inhibition of ^{51}Cr release was also obtained by addition of 25 g % bovine serum albumin to an ongoing lytic reaction (for example, experiment 2, Table 3).

The single most important criterion dictating molecular passage across a semipermeable membrane seems to be the molecule's effective diffusion radius (R_{ES}) calculated from the Stokes-Einstein equation²⁰. From values in the literature²¹ for the molecules used in this study, it would seem that dextran molecular weight 40,000 ($R_{\text{ES}} \sim 45\text{\AA}$) and bovine serum albumin of molecular weight 68,000 ($R_{\text{ES}} 35.5\text{\AA}$), by affording minimal significant inhibition of ^{51}Cr -protein release from cells bearing a Rb hole, represent the approximate initial dimensions of the T-cell-induced lytic lesion.

These findings show a striking similarity to the nature of the antibody induced complement lesion in erythrocytes¹² and in Krebs ascites tumour cells^{11,22}. In these cases too, macromolecular passage across a damaged (K permeable) cell membrane, could be prevented by maintaining the cells in non-penetrating protein²² or dextran solutions¹². For reasons advanced earlier, there is good reason to suppose that the complement system is not involved in the T-cell-mediated lytic lesion^{7,8}. The nature of the effector molecule remains, however, in doubt.

Currently, it seems reasonable to suppose that the initial lytic lesion in the target cell membrane is inserted during the contact between effector T lymphocyte and its homologous target cell. After this initial lesion, the continued presence of the lymphocyte is unnecessary²³, and the demise of the target cell results from its own inability to handle the augmented influx of water into its cytoplasm.

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CHRISTOPHER S. HENNEY

Department of Medicine,
Johns Hopkins University School of Medicine and
O'Neill Memorial Research Laboratories,
Good Samaritan Hospital, Baltimore, Maryland 21239

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Note added in proof: Seeman has recently claimed²⁴ that the approaches used here to estimate lesion size are invalid. He reports that the efflux of haemoglobin (Hb) from hypotonically lysed cells is suppressed by the presence of extracellular macromolecules, even when the size of the membrane lesion is much greater than the effective diffusion radius of the extracellular molecules. He argues that the macromolecules act simply by reducing the diffusion rate of Hb, a suppression which, he asserts, is dependent on the weight-concentration, and not the molecular weight, of the extracellular macromolecule. The latter assertion makes it unlikely that this is an adequate explanation of the phenomenon described here for dextrans of molecular weight 9,000 and 70,000, used at the same weight concentrations, have very different effects on ^{51}Cr -protein release (Fig. 1).

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Immunosuppression detected in pregnant mice by rosette inhibition test

ATTENTION has recently been drawn to the immunological mechanisms concerned in the nonrecognition of the antigenic status of the foetus during uterine life. Several workers¹⁻³ have demonstrated that lymphocyte transformation is suppressed during pregnancy. As the rosette inhibition test⁴ has been used to show that the lymphocyte activity *in vivo* is modified by immunosuppressive therapy following transplantation⁵, it suggests that this technique could also be used to investigate possible immunosuppression during pregnancy.

The rosette inhibition titre (RIT) was estimated as described previously⁶. Antilymphocytic serum (ALS) was produced against mouse spleen cells, and was tested for its effect on rosette formation between mouse spleen cells and human red blood cells (HRBC), (H.M., V.H., and G.J.A.C., unpublished). The HRBC were collected fresh each day from the same donor. The ALS was diluted to 1×10^6 and then serially diluted and tested in the range 1×10^6 to $32,768 \times 10^6$. The spleen cells used were from Quackenbush mice aged between 8 and 12 weeks. The RIT was recorded as the highest dilution of ALS at which the number of rosettes formed was 75% or less of the control (Hank's solution without ALS). The RIT of the ALS produced in this series showed significant correlation with the skin graft survival time.

The results of the RIT performed in two mice, a non-pregnant and a pregnant female respectively, are illustrated in Fig. 1, in which the rosette formation as a percentage of the control (without ALS) is plotted against the dilution of ALS. There was a marked difference in RIT, that in the nonpregnant female being 16×10^6 and in the pregnant female $8,192 \times 10^6$. The RIT of ten pregnant females and the normal group of five males and six non pregnant females is illustrated in Fig. 2. The logarithmic mean of $\text{RIT} \times 10^{-6}$ (to base 2) in the normal group was 3.2 (s.e., 0.42). The RIT of the pregnant group ranged from 32×10^6 to $8,192 \times 10^6$ (log mean 10.2 s.e., 1.05). There was no significant difference between the mean of the titres of the group containing five males and the group containing six nonpregnant females. There was a significant difference between the mean of the titres of the non-pregnant group (five males plus six females) and the pregnant group (ten females) $P < 0.001$. The mean number of spontaneous rosettes formed in the control with spleen

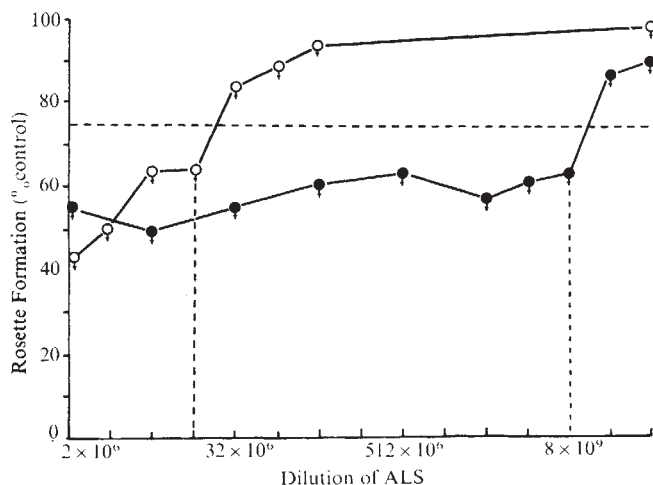


Fig. 1 Rosette formation (as % of the control) with lymphocytes from a pregnant (●) and a nonpregnant (○) mouse plotted against ALS dilution, illustrating the high RIT during pregnancy.

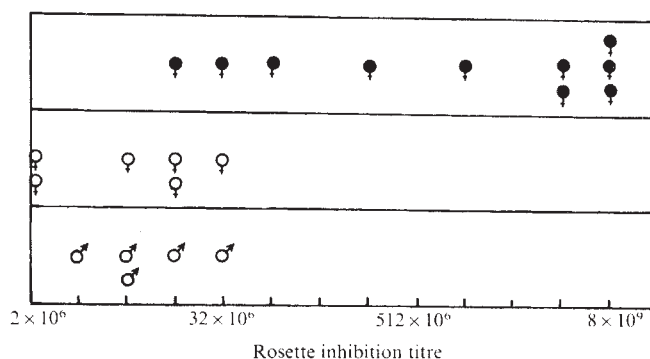


Fig. 2 RIT in male (♂), nonpregnant female (♀), and pregnant female mice (●), showing rise in RIT in some pregnant females.

cells from five male mice and six non pregnant female mice was 31.2 (s.e., 2.2) rosettes per 1,500 spleen cells, and the mean number in the control with spleen cells from ten pregnant females was 33.9 (s.e., 2.7) rosettes per 1,500 spleen cells. There is no significant difference between the number of rosettes formed in these two groups.

To investigate this wide range of RIT in pregnant mice and to follow an apparent change in relation to the stage of pregnancy, mice were killed at different stages after mating and the stage of pregnancy recorded as the crown-rump length of the foetus. A plot of RIT of pregnant mice against crown-rump length of foetus (Fig. 3) indicates

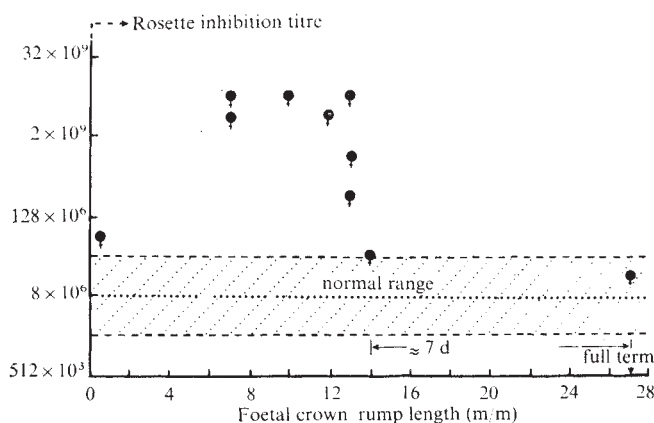


Fig. 3 RIT plotted against stage of pregnancy as measured by crown-rump length, showing a rise in RIT during pregnancy and a fall to normal before delivery.

that the RIT starts to rise early in pregnancy, and then drops to within the normal range approximately 7 d before delivery. The RIT of a mouse tested at full term lay within the normal range.

Our findings support the work of others¹⁻³ that the immunological competence of maternal lymphocytes is modified during pregnancy. They found that the phytohaemagglutinin-induced transformation was reduced. Finn *et al.* raised the question whether this was due to a central suppression of the T cells in pregnancy or to fewer T cells present to be stimulated. Our findings support the first of these two hypotheses. If there were fewer T cells to be stimulated then the number of spontaneous rosettes formed in the pregnant group would be less than the number formed in the nonpregnant group. We have found that this is not so, that there was no significant difference between the means of the results obtained from each group.

Contractor *et al.*³ have concluded from their investigations that HCS and HCG, produced by the placenta, are either binding or competing for the site of PHA stimulation. From our investigations, it seems likely that a similar reaction is occurring. The number of spontaneous rosette-forming cells in pregnancy is not decreased but the number of combining sites on these cells, available for the immunosuppressive activity of the ALS, is decreased. This is shown by the rosette forming activity of these cells being inhibited by much higher dilutions of ALS than those cells from nonpregnant animals. This indicates that the blocking of binding sites on the lymphocyte *in vivo* impairs its immunological competence. We have shown that the RIT begins to return to within normal limits at the beginning of the third week of pregnancy and this lends support to the theory of Contractor *et al.*, that immunorejection may play a part in stimulating parturition.

HALLE MORTON
VALERIE HEGH
G. J. A. CLUNIE

Department of Surgery,
University of Queensland at the
Princess Alexandra Hospital,
Brisbane, 4102 Australia

Received November 20, 1973; revised February 27, 1974.

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Stabilisation of interferons by defensive reversible denaturation

THE stabilities of interferons vary widely, depending on their source and degree of contamination. Even relatively stable interferons seem to become labile as various degrees of purity are attained¹. In the absence of a more suitable means of stabilisation, some investigators rely on addition of extraneous protein to stabilise interferons during purification², thus, a method for stabilising interferons would have considerable value.

The commonly encountered inactivations of interferons (deterioration on storage, repeated freezing and thawing, surface inactivation by shaking or frothing, or exposure to heat) occur under conditions where alterations of primary structures of protein are not likely; therefore, such inactivations probably result from denaturations involving only disruptions in secondary and tertiary structures. Theoretically such denaturations should be reversible.

Denatured proteins are more likely to return to native conformations if their amino acid sequence is in a linear random coil³, that is, a primary structure in which all noncovalent bonds and all cross links (disulphide bonds) have been broken. Since some interferons have been reported to contain disulphide bonds^{1,4}, attainment of a linear polypeptide should require reduction of such groups. As with some interferons, unfolding of the native protein is required to make cross linkages accessible to attack, an agent to disrupt noncovalent bonds would be required. Presumably, interferon polypeptides maintained in

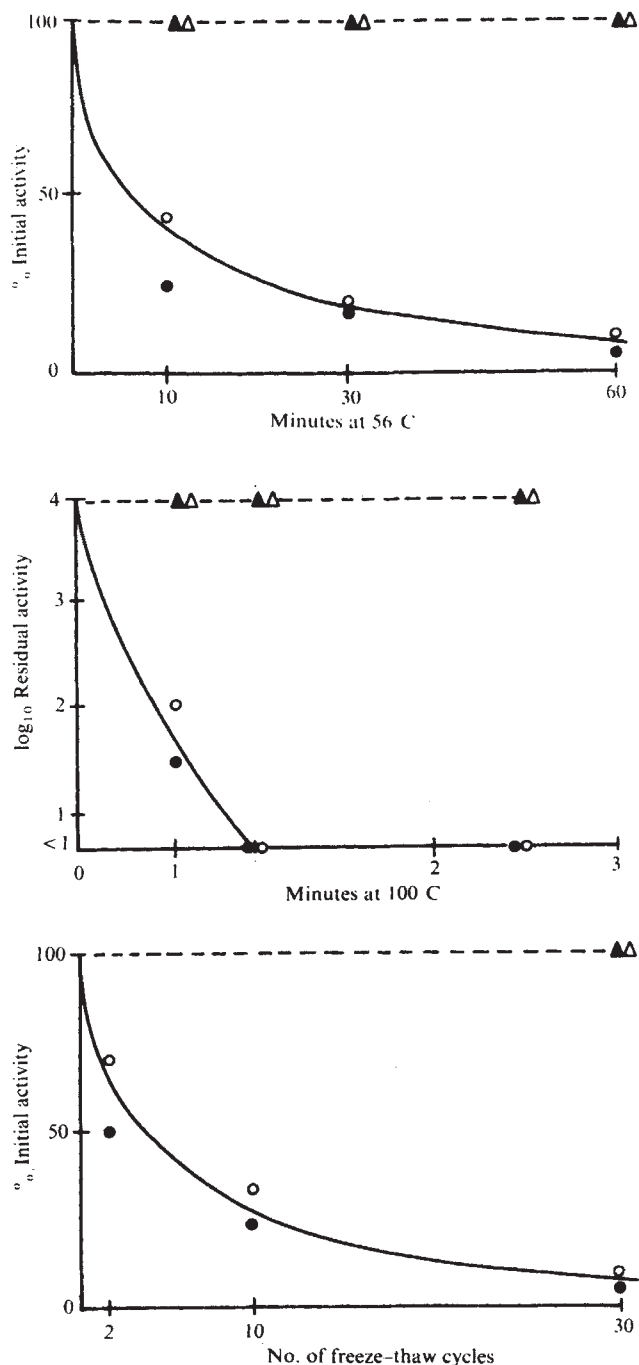


Fig. 1 Stabilisation of interferons by 'defensive' reversible denaturation. Interferons were subjected to the indicated treatment in their native state (○, ●) or in the 'defensively' denatured state (△, ▲). ●, ▲, Mouse interferon; ○, △, human interferon.

such a conformation should be stabilised against inactivation by procedures not inducing covalent changes in the primary structure. To maintain interferon in such a state, we selected a system consisting of urea, 2-mercaptoethanol, and the sodium dodecyl sulphate (SDS). The first two agents should provide random coils, which would theoretically be reversibly denatured, but in practice, oxidation of sulphhydryl groups or exchange of sulphhydryl and disulphide bonds are almost unavoidable upon their removal⁵. SDS should, however, defend the reactive groups against interactions⁵.

Mouse interferon prepared in L₉₂₉ cells induced with Newcastle disease virus was acidified to pH 2, extraneous proteins were precipitated by addition of ammonium sulphate to 20% saturation at 20°C, and clarified supernatant fluid was adjusted to pH 7.2 by dialysis against 0.01 M Tris HCl buffer. Prepara-

Table 1 Labilities of unfolded and reduced interferons under conditions allowing intra and intermolecular interaction

	Reagent	log ₁₀ Activity		
		Initially	After 56° C 30 min	After 100° C 2 min
Mouse interferon	None	3.8	3.0	< 1
	Mercaptoethanol (1.4 × 10 ⁻² M)	3.5	1.5	< 1
	Urea (5 M)	3.3	1.0	< 1
	Mercaptoethanol + urea	3.0	< 1	< 1
	None	4.0	3.5	< 1
Human interferon	Mercaptoethanol	3.8	1.5	< 1
	Urea	3.5	1.5	< 1
	Mercaptoethanol + urea	2.8	< 1	< 1

tions had specific activities of about 10⁶ units per mg protein. Human interferon produced in human diploid fibroblast cells induced with poly(I)-poly(C) was dialysed against 0.01 M Tris HCl buffer, pH 7.2.

The labilities of mouse and human interferons to various denaturation procedures were remarkably similar (Fig. 1). Heat inactivation kinetics of mouse interferons were independent of the purity of the preparation: preparations with about 10⁴, 10⁵, 10⁶ or 10⁷ units per mg protein showed identical inactivation kinetics.

The interferons were only slightly affected by either urea or mercaptoethanol at room temperature and exhibited the same 100° C heat lability in their presence as was seen in their absence; at 56° C, these agents markedly accelerated the inactivation rate (Table 1). The combination of urea and mercaptoethanol caused a greater loss of initial activity and, again, had no stabilising effect on the residual activity. Interferons denatured under these conditions seemingly allow intramolecular and intermolecular interplay.

Addition of SDS to denatured, reduced proteins might be expected to defend against this random, nonselective interplay of reactive groups which presumably contributed to the seemingly irreversible denaturation. Reduced proteins dissociated by urea and SDS seem to stay dissociated in SDS after removal of the unfolding and reducing agents^{6,7}. Both human and mouse interferons in the SDS-'defended' state were completely stable to heat and freeze-thaw cycles (Fig. 1). 'Defensive' reversible denaturation was achieved by making the interferons 3.5 × 10⁻³ M SDS, 1.4 × 10⁻² M 2-mercaptoethanol and 5 M urea; in other experiments 3.5 × 10⁻² M SDS, 1.4 × 10⁻¹ M 2-mercaptoethanol and 5 M urea gave identical results. Presumably, interferons in this condition are converted to linear peptide chains and after removal of urea, mercaptoethanol and excess SDS by dialysis, the SDS-interferon remains dissociated and returns to an active conformation when incubated with protein-containing medium. Significantly, addition of SDS alone to the interferons reduced their activities by between 60 and 90%, but the residual activity was stabilised in the presence of SDS alone. This suggests that interferons can renature to some extent from configurations that have cross-linked peptide chains, but can renature more efficiently from the linear polypeptide supposedly attained in SDS, urea and mercaptoethanol. Alternatively, this could suggest there are two distinct molecular species in these interferon preparations and that the major component does not renature after treatment with SDS alone, but does so if it is unfolded in SDS and mercaptoethanol, while the minor component is able to unfold in SDS alone and to renature. Since there are no known protein aggregates linked by noncovalent bonds that survive treatment with SDS at 100° C for 1 min (ref. 5), this stabilising process allows the determination of the molecular species of dissociated, denatured and reduced interferons by SDS-polyacrylamide gel electrophoresis and the relative biological (antiviral as against nonantiviral)⁸

properties resident in the renatured molecular species of interferons.

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WILLIAM E. STEWART II
ERIK DE CLERCQ
PIERRE DE SOMER

Department of Virology,
Rega Institute for Medical Research,
University of Leuven,
Leuven, Belgium

Received December 21, 1973; revised February 20, 1974.

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Surface H-2 antigen concentration requirement of somatic hybrid cells for IgM-mediated cytotoxicity

THE immune response following grafting of allogeneic or semiallogeneic cells in the mouse is characterised by the development of cytotoxic effector lymphocytes and/or antibodies which become cytotoxic *in vitro* in the presence of complement. I have studied the kinetics of the humoral response in DBA/2 inbred mice injected with semi-allogeneic somatic cell hybrids and shown that these hybrid cells were unexpectedly resistant to the complement-dependent cytotoxic action of antisera from the initial peak of the biphasic response. The allogeneic parental cells were however highly sensitive to cytotoxicity under the same experimental conditions. This serological response seems to be directed against the H-2 alloantigens expressed on both the allogeneic parental cells and on the semiallogeneic hybrid cells derived from it. In my attempts to understand this phenomenon, I found that the mouse alloantibodies of the IgM and IgG classes have different efficiencies when tested in the cytotoxic test on cells having a low H-2 antigen concentration on their plasma membrane.

Sera from the first peak (which extended from days 6-9 after injection of Y2C hybrid cells exhibited a clear cytotoxic effect when tested against the allogeneic parental cell C1 1D (sera 1, Table 1). The mean titre, defined as the dilution of serum at which 50% of chromium was released into the supernatant, was 1:32. But when labelled Y2C cells were the target cells, most of these sera did not liberate significantly more chromium than the chromium release background. (Background release was measured in controls where Y2C cells were incubated with complement alone, or with complement and serum from non-immunised DBA/2 mice). Sera from hyperimmunised mice, however, were equally effective against both C1 1D and Y2C cells. The mean titre for C1 1D was 1:128; for Y2C cells, 1:256 (sera 2, Table 1).

This response was directed mainly against the H-2^k transplantation antigens as was demonstrated by immunoabsorp-

Table 1 Cytotoxic activity of antisera as a function of antibody size and target cell use

Antisera group	Cell injected	Immunised strain	H-2 antigens against which antibodies were made	Antibody size	Target cell	Mean antisera titres
1*	Y2C	DBA/2	H-2 ^k	19S	C11D	1:32
					Y2C	< 1:4
2†	YC2	DBA/2	H-2 ^k	19S	311D	1:128
				7S	Y2C	1:256
3	C3H lymphocytes	BALB/c	H-2 ^k	7S	C11D	1:2,048
	DBA/2 lymphocytes	C3H	H-2 ^d	7S	Y2C	1:2,048
4	(C3H × DBA/2)F1 lymphocytes	B10.M	H-2 ^k	19S	L1210	1:2,048
			H-2 ^d		Y2C	1:2,048
5					L1210	1:4
					C11D	1:4
					Y2C	1:4

* Sixteen individual antisera were tested.

† Produced by nine weekly i.p. injections of 3×10^6 Y2C cells. Sera from eight different mice were tested.

For groups 1 and 2 Y2C hybrid clone was derived from the fusion of L1210-R lymphocytic leukaemia cells of DBA/2 mouse origin (H2^d) (ref. 1) with LM(TK⁻) C1 1D cells, a subclone of C3H mouse L cell line (H2^k) (ref. 2). Cells were fused using ultraviolet-inactivated Sendai virus³. The isolation and culture conditions for *in vitro* growth of the hybrid clones have been described elsewhere⁴. C1 1D and L1210 parental and Y2C hybrid cells were propagated in culture in Dulbecco's modified Eagle's medium with 10% heat-inactivated calf serum. The cells (except L1210 cells which grew in suspension cultures) were dispersed with 0.02% versene in Dulbecco's phosphate buffered saline (PBS) rinsed with PBS and suspended in Dulbecco's medium. Three month old DBA/2 mice were inoculated subcutaneously with 3×10^6 viable Y2C hybrid cells and bled daily; sera were stored at -20°C until tested. Their activity was measured as complement-dependent cytotoxicity by the chromium release test⁵. One millilitre of a target cell suspension (5×10^6 viable cells ml⁻¹) was incubated at 37°C for 30 min with 200 μCi sodium chromate (⁵¹Cr) and washed exhaustively with Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal bovine serum. 1×10^6 labelled cells were incubated with serial two-fold dilutions of antisera in the presence of rabbit serum (diluted 1:10) as a source of complement, in a final volume of 0.25 ml. At the end of the incubation period (1 h at 37°C in a 5% CO₂ atmosphere), 1.75 ml of ice-cold PBS was added to each tube, and after centrifugation, 1 ml of the supernatant was assayed for released chromium in a well-type gamma radioactivity counter.

tion experiments. Some cytotoxic activity remained after the absorption of hyperimmunised DBA/2 mice sera with C3H mouse splenic lymphocytes; this residual cytotoxicity might be directed against the virus-induced antigens on the L cell line⁶, and in hybrid cells in which L cell derivatives (C1 1D) were partners⁷⁻⁹.

The sizes of the antibodies for the initial and second peak were studied by fractionating the sera at 4°C on a Sephadex G-200 column (1.5×60 cm) equilibrated with PBS. Fractions were concentrated by B 15 Minicon ultrafiltration (Amicon) and tested immediately for cytotoxicity against ⁵¹Cr-labelled cells. Only 19S antibodies could be found in the initial peak of the immune response; both 19S and 7S antibodies of approximately equal cytotoxic titres coexisted in the sera from the hyperimmunised mice. When tested for sensitivity to 2-mercaptoethanol, the 19S fraction completely lost its activity but the cytotoxicity of the 7S fraction was only slightly reduced.

In another experiment, both parental and hybrid cells were titred by alloantisera specifically directed against the H-2^d and the H-2^k transplantation antigens. Antisera, made in BALB/c and C3H inbred mice by hyperimmunisation with lymphnode and splenic lymphocytes of C3H and DBA/2 origin, respectively, were tested against target cells. Fractionation of these sera showed that the bulk of the activity was due to molecules of the 7S class. The titres were 1:2,048 when tested against the lymphocytes used in the immunisation. The same high activity was found when titred against the allogeneic parental cell and the Y2C somatic hybrid (sera 3 and 4, Table I) demonstrating that, in accordance with other reports⁷⁻⁹, the hybrid clone expresses major histocompatibility antigens of both strains of mice from which the parental cells were derived and that Y2C cells were very actively lysed by 7S antibodies. Furthermore, when those antisera were tested on Y2C cells by the indirect immunofluorescence technique, a clear positive reaction was observed on 97% of cells.

Since some reports have stated that mouse 19S antibodies react preferentially with guinea pig complement¹⁰, the cytotoxic test with sera from group 1 was performed in the presence of fresh rat (diluted 1:4) and lyophilised guinea pig sera (1:2) as a source of complement. No improvement was found in ⁵¹Cr release when such complements were used instead of rabbit complement.

As antigen masking by sialic acid may occur in both normal cells and tumour cells^{11,12}, removing sialic acid from Y2C cell surfaces by neuraminidase might improve the cytotoxic activity of IgM. Five million labelled Y2C hybrid cells were incubated with 5 IU of *Vibrio cholerae* neuraminidase (Behringwerke) for 20 min at 37°C in 1 ml of PBS (pH 6.5). After washing, the viability was determined by trypan blue dye exclusion and cells were assayed for susceptibility to 19S-mediated cytotoxic effect. Neuraminidase-treated cells became more sensitive to the action of IgM (mean titres changed from < 1:4 to 1:8) presumably as the result of the re-exposure of antigenic determinants, with the concomitant increase in complement-fixing sites.

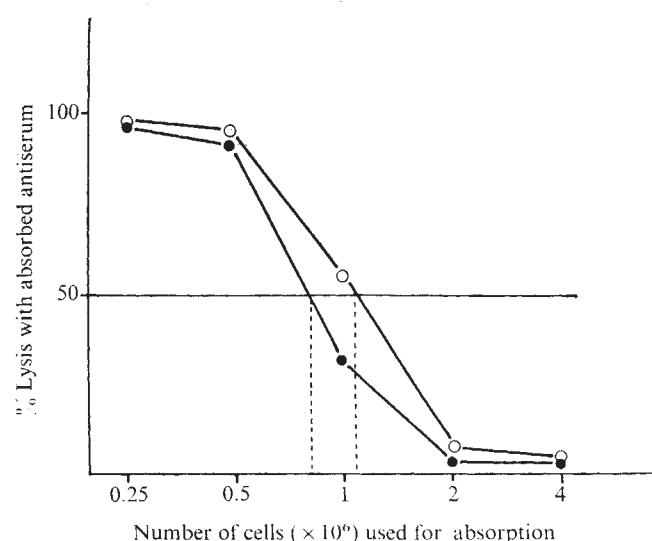


Fig. 1 Antibody binding capacity of C1 1D and Y2C cells. Serum 3, diluted to give a 90–95% cytotoxic activity was absorbed with increasing numbers of cells at 37°C for 30 min and then allowed to stand for 30 min at 4°C . After centrifugation, the remaining activity of the supernatant was tested against the appropriate sensitive cells. The values are based on the results of three experiments. ○, C1 1D; ●, Y2C

When target cells were tested against 19S antibodies from adult B10M inbred mice injected with 8×10^7 splenic cells of (C3H $\times$ DBA/2) F₁ hybrid mice and bled 4 d after the injection, a weak but uniform response was found (sera 5, Table 1) the increase in the number of target sites leading to an enhanced susceptibility to lysis in Y2C cells.

The quantitative differences in H-2^k antigen concentration on the plasma membrane from C1 1D and Y2C cells were then investigated by studying the absorption capacity of intact viable cells. Figure 1 shows that the H-2^k antigen concentration on C1 1D cells was approximately 1.5 times greater than on the hybrid cells. Diameters of both cell types were measured with an ocular micrometer (200 cells of each sample), and the ratio of surface area of Y2C cells to that of C1 1D fibroblasts was calculated to be 5:4 (i.e. 20% greater). It seems therefore that the H-2^k antigenic density on Y2C cells is about 55% of that on the C1 1D plasma membrane, if uniform surface smoothness is assumed.

An inverse relationship between the quantitative expression of H-2 and tumour-specific antigens has been demonstrated by some authors¹³⁻¹⁵. In somatic hybrid cells it is possible that all the antigenic determinants of both parental cells are expressed on the cell surface. In addition to the H-2^k antigenic complex and the L antigen, C1 1D express some group-specific murine leukemia antigens⁶. L1210 cells may contribute to the Y2C antigenic constitution with the H-2^d isoantigen complex and also with its tumour-specific transplantation antigens¹⁶. Thus there is competition for the plasma membrane surface in hybrid cells, resulting in a topographic exclusion of some antigens.

As the immune lysis of mouse leukemic cells by IgM is a 'one-hit' reaction¹⁷, the relative resistance of Y2C hybrid to the 19S antibody-mediated lysis might be due to the great difference between the number of complement-fixing sites and lytic sites available on the plasma membrane. In fact, 3,000 complement C1-fixing sites were necessary to generate one lytic site on the surface of a mouse leukaemic cell, the IgG being more effective in generating C1-fixing sites¹⁷. The restricted expression of H-2 antigens on the surface of Y2C somatic cell hybrids seems to be responsible for the lack of sensitivity to lysis mediated by IgM antibodies¹⁸. Moreover, a critically spaced repeating H-2 determinant on the plasma membrane must be required for the induction of complement-dependent cytotoxic effect.

Thus, caution should be exercised in interpreting results of studies carried out with the cytotoxicity assay on cell populations with a low density of transplantation antigens (that is, tumour-specific antigens) on the cell surface.

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NAZARIO RUBIO*

Institut de Recherches Scientifiques sur le Cancer,
94800-Villejuif,
France

* Present address: Instituto Jaime Ferrán de Microbiología, Joaquín Costa, 32-Madrid 6, Spain

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Subunit structure of HL-A antigens on cell surface

THE major human histocompatibility determinants, the HL-A antigens, are cell surface markers present on lymphocytes and most other nucleated cells (compare ref. 1). Recently, work from two laboratories has indicated that HL-A antigens, released from the cell surface by means of papain digestion, are composed of two polypeptide chains^{2,3}. The smaller of the two subunits seems to be invariant regardless of the antigenic specificity carried by the larger HL-A chain^{2,3} and occurs in human biological fluids⁴.

It had previously been demonstrated that β_2 microglobulin exhibits extensive amino acid sequence homologies with immunoglobulin heavy and light chains^{5,6} and that β_2 microglobulin is an integral part of the cell surface^{5,7,8}. In a search for its function we showed that β_2 microglobulin was one of the two HL-A polypeptide chains⁹. Independently, Nakamuro *et al.*¹⁰ demonstrated that the small HL-A chain shared some properties with β_2 microglobulin.

Here we present evidence that β_2 microglobulin and the alloantigenic HL-A chain are physically linked to each other on the cell surface of lymphocytes.

Purified lymphocytes were isolated from defibrinated blood by Ficoll-Isopaque gradient centrifugation^{11,12}. The blood was obtained from healthy blood donors with known HL-A phenotype. The cells were subjected to the microlymphocytotoxicity test¹³ with purified anti- β_2 microglobulin antibodies¹⁴, and cells from all donors were completely lysed regardless of their HL-A phenotype^{9,15,16}.

It was argued that if β_2 microglobulin and the alloantigen-carrying HL-A chain are indeed parts of the same molecule, antibodies directed against one of the polypeptide chains may interfere with the simultaneous binding of antibodies to the other chain. The results of experiments designed to test this possibility are summarised in Table 1. Fab' fragments¹⁷ against β_2 microglobulin could completely and specifically inhibit the cytotoxic action of bivalent anti- β_2 microglobulin antibodies. In control experiments, Fab' fragments against light immunoglobulin chains had no effect. Fab' fragments directed against various HL-A determinants could specifically inhibit the cytotoxic action of the corresponding bivalent antibodies but not if the anti-HL-A antibodies were directed against some other specificity. A number of monospecific anti-HL-A antibodies could not cause lysis of cells that had been pretreated with Fab' fragments against β_2 microglobulin (Table 1), whereas their cytotoxic activity was normal when tested against cells treated with Fab' fragments against immunoglobulin light chains. Cells derived from a doubly homozygous donor, that is, expressing only one serological specificity for each HL-A sublocus, were separately treated with Fab' fragments directed against one

Table 1 Effects of Fab' fragments on the lymphocytotoxic effect of anti- β_2 microglobulin and anti-HL-A antibodies*

Specificity of Fab' fragments†	Specificity of antibodies	Cytotoxic effect‡
-----	β_2 M	++++
β_2 Microglobulin	β_2 M	—
Light immunoglobulin chains	β_2 M	++++
-----	HL-A-1	++++
HL-A 1	HL-A 1	—
-----	HL-A 8	++++
HL-A 8	HL-A 8	—
HL-A 1	HL-A 8	++++
β_2 M	HL-A 1	—
Light immunoglobulin chains	HL-A 1	++++
β_2 M	HL-A 8	—
Light immunoglobulin chains	HL-A 8	++++
HL-A 1	β_2 M	++++
HL-A 8	β_2 M	+++
HL-A 1, 8	β_2 M	++

* The cells were incubated for 45 min at 37° C in Eagles' MEM with various concentrations of the Fab' fragments indicated, washed three times in the medium before being subjected to the cytotoxicity test¹³. The concentrations of the Fab' fragments and the cytotoxic antibodies used were chosen such that a two-fold dilution of the working solutions notably diminished their blocking or cytotoxic activity, respectively.

† The target cells used in the present experiment were from a donor homozygous for the HL-A antigenic specificities 1 and 8. In a number of identical experiments, results similar to those listed were obtained with anti-HL-A antibodies of specificities 2,7,10,11,27, W10, and W15.

‡ The cells were scored +++++ if more than 75%, +++ if more than 50%, ++ if more than 25%, + if more than 15%, and — if less than 15% of the cells were stained by trypan blue.

of the two HL-A specificities. A slight but significant reduction in the cytotoxic activity of anti- β_2 -microglobulin antibodies compared with untreated control cells was noted. If, however, the cells were simultaneously treated with a mixture of Fab' fragments directed against each of the two subloci gene products, the cytotoxicity was substantially reduced. Thus, β_2 microglobulin and the alloantigenic, larger HL-A polypeptide chain are located in close proximity on the cell surface.

Attempts were made to demonstrate simultaneous redistribution of β_2 microglobulin and the alloantigenic HL-A chain on the surface of purified lymphocytes to show that the two polypeptide chains are physically linked to each other.

Fluorescein isothiocyanate (FITC)-conjugated anti- β_2 microglobulin antibodies¹⁸ were incubated together with purified lymphocytes. Details of the materials used have been described elsewhere¹⁹ and the experimental protocol was essentially that of Kourilsky *et al.*²⁰. Anti- β_2 microglobulin fluorescence was present on more than 90% of the nucleated cells (compare Table 2). A majority of the cells had patches and a few displayed polar caps. If, however, the FITC-anti- β_2 microglobulin antibody-treated cells were incubated with goat-anti-rabbit immunoglobulin (GARIG) antibodies, rendered specific against the Fc portion of the molecule by thorough absorption with rabbit Fab' fragments, more than 70% of the stained cells exhibited fluorescent caps. FITC-anti- β_2 microglobulin Fab' fragments gave diffusely distributed fluorescence both in the absence and presence of GARIG. In control experiments with use of FITC-rabbit-anti-human immunoglobulin (RAHIG) antibodies and GARIG treatment, all fluorescent cells showed caps. This cap formation did not, however, induce any redistribution of FITC-anti- β_2 microglobulin Fab' fragments, thus establishing that β_2 microglobulin on the cell surface is quite independent from cell membrane-bound immunoglobulins.

FITC-conjugated anti-HL-A antibodies of different specificities gave rise to cells exhibiting patches or caps. The cap formation was more pronounced in the presence of RAHIG. Only diffuse fluorescence was evident on cells treated with anti-HL-A Fab' fragments whether GARIG had been added or not.

Attempts were made to induce patch and cap formation of

FITC-anti-HL-A Fab' fragments with successive treatment of the cells with anti- β_2 microglobulin antibodies and GARIG. The anti- β_2 microglobulin antibodies, obtained from rabbits immunised with free β_2 microglobulin, did not cause any redistribution of the diffuse fluorescence but rather seemed to displace a large proportion of the fluorescence from the cell surface. This could, however, be explained by the above results on the inhibition of the anti- β_2 microglobulin antibody-induced lymphocytotoxicity (Table 1). Anti- β_2 microglobulin antibodies were therefore obtained from a rabbit immunised with highly purified, papain-solubilised HLA antigens and isolated by immunosorbent purification²¹. These anti- β_2 microglobulin antibodies together with FITC-anti-HL-A Fab' fragments and GARIG induced caps in all experiments (Table 2).

Cells incubated with FITC-anti- β_2 microglobulin Fab' fragments, obtained from the rabbit anti-HL-A serum, as described above, were treated in succession first with anti-HL-A antibodies and then with RAHIG. A significant number of cells (> 60%) exhibited patches and with some anti-HL-A antibodies even caps (Table 2). In all experiments, however, a few cells always showed only a diffuse fluorescence.

These experiments clearly demonstrate that β_2 microglobulin and the alloantigenic HL-A polypeptide chain are physically linked on the lymphocyte surface.

As the β_2 microglobulin amino acid sequence is highly homologous to immunoglobulin light and heavy chains^{5,6}, it has been suggested that it is an evolutionary precursor to regular immunoglobulin polypeptide chains⁵. It now seems possible that at least part of the alloantigens and the immunoglobulins may have arisen from a common evolutionary origin^{5,22}.

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Table 2 Effects of various antibodies on the redistribution of the antigen-carrying HL-A chain and β_2 microglobulin on the cell surface.

FITC-conjugated component	Additive	Appearance of cell surface bound fluorescence†		
		Diffuse	Patches	Caps
Anti- β_2 M antibody	---	+	+	—
Anti- β_2 M antibody	GARIG	—	+	+
Anti- β_2 M Fab'	---	+	—	—
Anti- β_2 M Fab'	GARIG	+	—	—
Anti- β_2 M Fab'	RAHIG + GARIG	+	—	—
RAHIG	GARIG	—	—	+
Anti-HL-A 1 antibody	---	+	+	+
Anti-HL-A 8 antibody	---	+	+	+
Anti-HL-A 1 antibody	RAHIG	—	+	+
Anti-HL-A 8 antibody	RAHIG	—	—	+
Anti- β_2 M-Fab'	Anti-HL-A 1 antibody + RAHIG	+	+	—
Anti- β_2 M Fab'	Anti-HL-A 8 antibody + RAHIG	+	+	+
Anti-HL-A 1 Fab'	Anti- β_2 M antibody + GARIG	—	+	+
Anti-HL-A 8 Fab'	Anti- β_2 M Antibody + GARIG	—	—	+

Approximately 2×10^6 cells were incubated for 30 min at 37° C in 0.2 ml of Eagles' MEM containing an appropriate concentration of the FITC-conjugated component (1–4 mg ml⁻¹). At the end of the incubation period, the cells were washed three times with the medium, and examined in a Leitz Ortholux microscope equipped with a HBO 200 W lamp. The Ploem-type vertical illuminator and immersion objectives were used. If further treatment of the cells was required they were incubated with antibodies (3–8 mg ml⁻¹) in Eagles' MEM for another 30 min, washed three times with the medium, examined in the fluorescence microscope or incubated with GARIG or RAHIG (3–5 mg ml⁻¹) for another 30 min in Eagles' MEM before evaluation of the cell-surface bound fluorescence.

† If more than 20% of the fluorescent cells showed a certain type of distribution of the label, they were scored as positive.

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After submission of this manuscript, two papers with data similar to ours have appeared^{23,24}.

LARS ÖSTBERG
J. BERTIL LINDBLOM
PER A. PETERSON

The Institute of Medical Chemistry
and the Blood Center,
University of Uppsala,
Uppsala, Sweden

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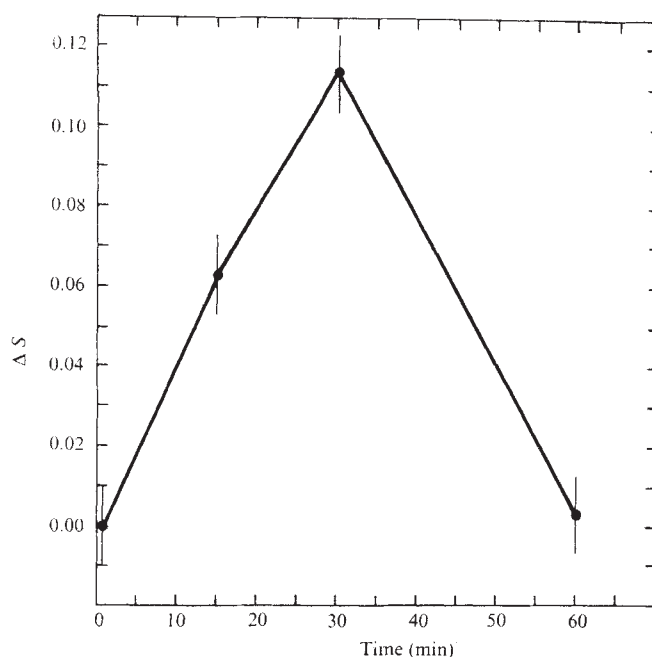


Fig. 1 The effect of an optimal mitogenic dose of PHA-P (Difco, $7.5 \mu\text{g ml}^{-1}$) on the fluidity of the human lymphocyte membrane at various times after addition of the mitogen.

$$\Delta S = S_{\text{control}} - S_{\text{PHA}} \text{ and } S_{\text{control}} = 0.52.$$

In lymphocytes recent observations suggest that mitogens also induce an early increase in levels of cyclic GMP¹⁰. This nucleotide increases more than ten-fold within 5–20 min of the addition of PHA or con A, followed by a decline to basal levels within 1 h. Other mitogenic agents such as phorbol myristate acetate⁸ and high concentrations of insulin⁹ also increase considerably the levels of cyclic GMP in lymphoid cells. It has been suggested that this increase is part of the signal to initiate cellular proliferation. After binding of mitogenic lectins other early changes in the plasma membrane occur; examples are increased endocytosis¹¹ and incorporation of orthophosphate into phosphatidyl inositol¹².

According to our membrane model^{3–5}, lymphocyte mitogens which produce a transient elevation of cellular cyclic GMP should fluidise membrane lipids. The data reported here support this model.

Human peripheral blood lymphocytes, separated using a Ficoll-Hypaque density gradient, were 90% free of contaminating monocytes, erythrocytes and granulocytes. Mouse lymphocytes were obtained from CBA/Ht6 strain spleen cells which were minced and washed three times in buffered saline. The fluid characteristics of the plasma membrane of lymphocytes were probed with the spin label sodium 6-(4',4'-dimethylloxazolidinyl-N-oxyl)-heptadecanoate³. A 0.3 mM solution of the label in buffered saline was incubated with cells for 30 min at 37° C. The cells were then washed five times to remove excess label¹³. The electron paramagnetic resonance (EPR) spectra were recorded at 33° C with a Varian E-3 spectrometer.

The EPR spectra of the spin-labelled fatty acids in natural membranes indicate that the labels have two important characteristics: (1) they undergo rapid anisotropic motion about an axis approximately parallel to the fatty acid chain with a correlation time of $<10^{-7} \text{ s}$ ^{3,14–18} and (2) they undergo rapid lateral diffusion with a diffusion constant of $10^{-8}–10^{-7} \text{ cm}^2 \text{ s}^{-1}$ (refs 19–22). Since it is improbable that such rapid axial motion or rapid lateral diffusion could occur if the labels were bound to protein, these characteristics suggest that the labels are in a phospholipid bilayer-like environment. While the lateral

Evidence that mitogenic lectins induce changes in lymphocyte membrane fluidity

MITOGENIC plant lectins, such as phytohaemagglutinin (PHA) and concanavalin A (con A), are useful tools to study the control of cell proliferation in lymphocytes. Recent evidence suggests that these mitogenic lectins trigger mitogenesis at the cell membrane^{1,2}. It is likely that an understanding of the mechanisms by which these lectins stimulate lymphocytes will provide important clues to more general problems of the regulation of proliferation in eukaryotic cells.

A model has been proposed to explain how modulation of membrane structure may influence cell growth. This model suggests that changes in the fluid characteristics of membrane lipids affect a variety of membrane activities^{3–5}. Studies in isolated membranes have shown, for example, that fluidisation of membrane lipids is associated with activation of Na^+ , K^+ -ATPase⁶. *In vitro* studies have also correlated a fluid state of membrane lipids in transformed cells with high cellular levels of cyclic GMP and low levels of cyclic AMP and ordered membrane lipids in contact inhibited cells with low levels of cyclic GMP and high levels of cyclic AMP^{4,6–9}.

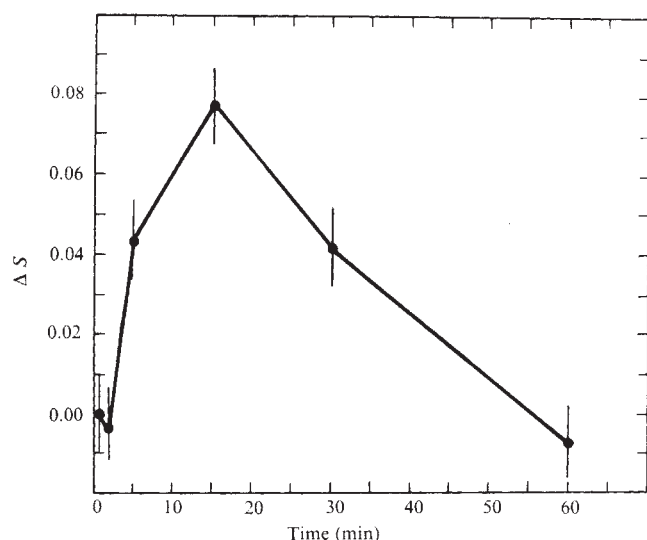


Fig. 2 The effect of con A (Difco, $3 \mu\text{g ml}^{-1}$) on the fluidity of the plasma membrane of mouse splenic lymphocytes at various times after addition of the Mitogen. $\Delta S = S_{\text{control}} - S_{\text{con A}}$ and $S_{\text{control}} = 0.52$.

diffusion of surface antigens in mouse-human heterokaryons throughout the cell membrane requires about 40 min²³, that for spin labels of fatty acids should require about 1 s.

Order parameters, S , were calculated from the EPR spectra. Values of S near 1 correspond to an ordered state of the membrane lipids while values near 0 correspond to a fluid state²⁴. With the label used in this study, a $\Delta S = 0.03$ would be equivalent to a change in membrane fluidity produced by a 6–10° C temperature change³.

The data in Figs 1 and 2 clearly demonstrate that treatment of human peripheral lymphocytes with mitogenic concentrations of PHA and treatment of mouse splenic lymphocytes with mitogenic concentrations of con A produce a fluidisation of membrane lipids. Both human and mouse lymphocytes showed maximal fluidisation between 15 and 30 min. The dose-response curve for membrane fluidisation also corresponds well with the dose-response curve for the mitogenic effect of con A (Fig. 3). In contrast, a nonmitogenic lectin, wheat germ agglutinin, had no effect on membrane fluidity.

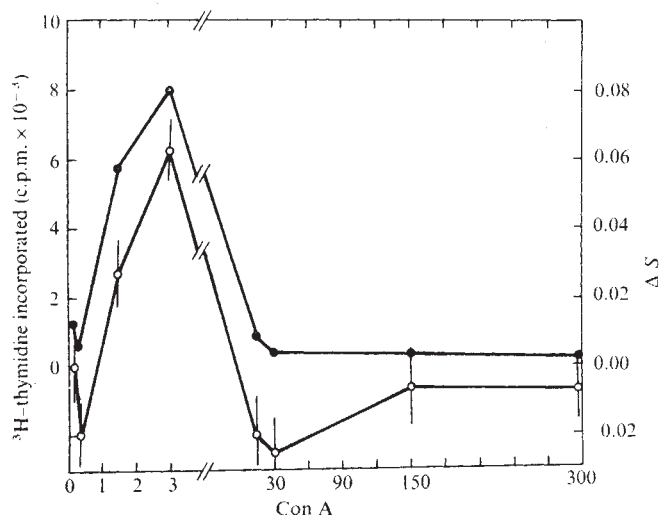


Fig. 3 The dose-response curves for the mitogenic effects of con A (●) and the membrane fluidising effect of con A for a 20 min incubation (○) with mouse splenic lymphocytes. Mitogenesis was determined by incorporation of ³H-thymidine into acid-insoluble precipitate after 72 h of incubation.

Studies are in progress to define precisely the location and distribution of the label in lymphocyte membranes, and to examine other mitogenic agents to determine whether critical membrane fluidisation is a prerequisite for mitogenesis in general. The data presented here demonstrate, however, that only mitogenic concentrations of plant lectins produce an early fluidisation of lymphocyte membranes and that the time of membrane fluidisation closely parallels the increase and decrease of cyclic GMP levels in stimulated lymphocytes¹⁰. It has been suggested that the increase of cellular cyclic GMP levels is a signal for cellular proliferation^{7–10} and our data suggests that early membrane fluidisation may be equally important in the triggering process.

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RONALD E. BARNETT
ROBERT E. SCOTT
LEO T. FURCHT
JOHN H. KERSEY

Departments of Chemistry and
Laboratory Medicine and Pathology,
University of Minnesota,
Minneapolis, Minnesota 55455

Received February 19, 1974.

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Hb K Woolwich the cause of a thalassaemia

In general terms, heterozygotes for β thalassaemia have a moderate degree of microcytosis associated with an elevated red cell count, a reduced mean cell volume (MCV) and mean cell haemoglobin (MCH) and a normal, or near normal, mean cell haemoglobin concentration (MCHC). The chief diagnostic parameter of the heterozygous state is, however, the elevated Hb A₂ level which accompanies the condition. This does not occur in other haemoglobinopathies with the exception of those associated with unstable haemoglobins and some heterozygotes for Hb S (ref. 1) and Hb C (ref. 2). At the molecular level β thalassaemia is associated with an imbalance of globin chain synthesis during erythroid cell maturation, as a result of defective β chain synthesis. The extent of the defect parallels the severity of the thalassaemia, ranging from a reduction in the amount of product from the affected β chain locus (β^+ thal) to no product (β^0 thal). In all cases of β^+ thal so investigated, the β chains produced by the affected gene have been indistinguishable from normal β^A chains.

We here present evidence which compares the inheritance of the β chain gene of Hb K Woolwich (Hb KW) with the inheritance of a β^+ thalassaemia. Also, the interaction of this thalassaemia with an α thalassaemia is demonstrated in a patient of West Indian origin.

Hb KW, first described in a West Indian family, is a stable haemoglobin variant³. Table 1 gives the pattern of haemoglobins in seven heterozygotes for Hb KW characterised in this laboratory. The amount of Hb KW is always proportionately less than the other adult haemoglobin (30–40%) and the Hb A₂ is higher than normal in six of the seven cases. The R family (Fig. 1) was available for more detailed haematological investigations. The propositus I-1 had the classical features of β thalassaemia trait; a moderate microcytosis and a high Hb A₂ level in the red cells (Table 1). Investigation of the abnormal haemoglobin identified it as Hb K Woolwich, although β -chain variants usually exceed 50% of the total haemoglobin in double heterozygotes for β thalassaemia and a β -chain variant^{2,4}. Nevertheless, the pattern was repeated in members of the family who had inherited the β^{KW} chain gene; they all had high levels of Hb A₂ per red cell (Table 1). The doubly abnormal heterozygote, (Hb KW+Hb S), II-3, had an exceptionally high Hb A₂ but the difference can be accounted for by the additional inheritance of the β^S chain gene, which is often associated with marginally higher levels of Hb A₂ per cell than normal¹ (Table 1).

The conclusion from these observations is that inheritance of the β^{KW} chain gene is associated with a mild β thalassaemia, that is the β^{KW} chain locus is a β^{KW+} thalassaemia gene. This is the first example of a β^+ thalassaemia in

which the product of the affected locus is distinguishable from the normal β^A chain.

Since the normal course, in suspected cases of thalassaemia, is an investigation of the *in vitro* biosynthesis of the globin chains, a reticulocyte-rich preparation of red cells was obtained from the blood of the propositus. The results of *in vitro* incorporation of ³H-leucine into the α^A , β^A and β^{KW} chains isolated from the whole globin of these red cells are summarised in Fig. 2. The incorporation of radio-isotope into all chains was linear over the incubation period of 60 min. The total incorporation of ³H-leucine into the β^{KW} chain was always much less than into the β^A chain and averaged less than 50% of the incorporation into the β^A chain (Table 2). The amount of Hb K Woolwich found in the blood of the propositus is therefore an accurate reflection of the amount of this chain synthesised during erythroid cell maturation. This fulfills another criterion required by the definition of β thalassaemia; namely the demonstration of defective synthesis of globin chains by one of the β chain loci.

Table 2 *In vitro* biosynthesis of α , β^A and β^{KW} chains in reticulocytes from propositus

Incubation Time (min)	3	6	9	15	30	60	Mean
$\beta^{KW}/\beta^A + \beta^{KW}$	0.29	0.36	0.34	0.31	0.30	0.30	0.30
α^A/β^A	1.30	1.20	1.20	1.10	1.00	1.10	1.20
$\alpha^A/\beta^A + \beta^{KW}$	0.93	0.80	0.78	0.79	0.73	0.74	0.79

The ratios correspond to the relative amounts of each chain synthesised based on the total incorporation of ³H-leucine into the individual chains during *in vitro* incubation of the reticulocytes.

A correlation between the mutation in the β^{KW} Woolwich chain β 132 (H10) Lys-Gln and the lesion responsible for the amount synthesised is important since this could confirm the predictions on one possible cause of β thalassaemia based on a "structure-rate hypothesis"⁵. There are several observations in favour of this explanation: (1) Hb K Woolwich is the only known haemoglobin variant resulting from a mutation at H10 Lys; (2) with the exception of the frog, where it is alanine, in all species so far examined, lysine (H10) is an invariant residue in all α , β^0 and myoglobin chains^{6,7}; and (3) a myoglobin variant has been characterised in which H10 Lys is replaced by a residue of asparagine and the amount of the myoglobin variant in muscle extracts is approximately 30% of the total myoglobin⁸.

It is possible that the tRNA coding for the β H10 glutamine is in short supply. This would introduce a partial block at position H10 and decrease the rate of translation of the β chain mRNA by the ribosomes. This possibility was investigated by comparing the rate of assembly and termination of the β^A and β^{KW} chains by a modification of the method of Dintzis⁹ (Table 3 and Fig. 3). From Fig. 3,

Table 1 haemoglobin pattern and haematological data of the R family and other heterozygotes for Hb KW

Case	% Total haemoglobin				Haematological data				
	Hb KW	Hb S	Hb A ₂	Hb F	Hb (g per 100 ml)	RBC ($\times 10^6 \mu l^{-1}$)	MCH (pg)	MCV (fl)	Hb A ₂ (pg per cell)
TG	39.2	—	4.8	—	—	—	—	—	—
IB	40.9	—	2.7	—	—	—	—	—	—
T	37.2	—	3.8	—	—	—	—	—	—
RI-1	28.4	—	5.1	0.7	14.3	6.2	23.1	69	1.2
RI-2	—	32.4	3.1	0.7	16.0	5.9	26.6	77	0.9
RII-1	—	—	2.9	0.6	15.1	6.6	22.6	69	0.7
RII-2	38.0	—	4.1	1.8	13.2	4.8	27.4	79	1.1
RII-3	40.0	54.2	5.2	0.6	16.0	7.0	22.6	66	1.2
RII-4	34.2	—	3.9	—	16.3	5.6	28.6	81	1.1
Normal range			2.5–3.5	< 0.8					0.75 (ref. 4)

The haemoglobins were quantitatively estimated by DEAE Sephadex chromatography¹⁵. Globin prepared from Hb KW (ref. 6), was separated into the individual chains by ion exchange chromatography on CM 23 cellulose¹⁷. The β^{KW} chain was characterised as β 132 (H10) Lys-Gln by fingerprinting the tryptic peptides, elution, hydrolysis and amino acid analysis of the abnormal peptide³. Haematological data were obtained from a Coulter S counter.

Table 3 Specific activity of leucine residues in the tryptic peptides of the β^A and β^{KW} chains after 6 and 9 min incubation with ^3H -leucine

Tryptic peptide (Tp)	^3H -leucine content (d.p.m.)				^{14}C -leucine content (nmol)				Specific activity d.p.m. per 100 nmol			
	β^A_6	β^{KW}_6	β^A_9	β^{KW}_9	β^A_6	β^{KW}_6	β^A_9	β^{KW}_9	β^A_6	β^{KW}_6	β^A_9	β^{KW}_9
1 (1)	70.9	49.3	100.2	52.5	129.4	97.4	104.9	58.6	54.8	50.6	95.5	89.6
2 (1)	—	76.3	76.6	70.9	—	139.9	82.4	75.8	—	54.5	93.0	93.5
3 (1)	80.3	83.1	170.3	—	144.7	144.3	174.7	—	55.5	57.5	97.5	—
4 (2)	90.3	—	—	96.2	161.1	—	—	106.8	56.1	—	—	90.1
5 (1)	86.7	52.5	92.8	80.4	157.9	93.6	97.9	84.2	54.9	56.1	94.8	95.3
9 (4)	256.2	153.7	252.5	218.5	444.6	257.3	267.2	236.8	57.8	59.7	94.5	92.3
11 (1)	—	61.7	110.7	—	—	99.1	112.4	—	—	62.3	98.5	—
12a (2)	103.7	—	142.3	64.5	177.6	—	141.6	67.9	58.4	—	100.5	95.0
12b (1)	74.0	59.8	110.3	66.8	125.3	97.9	115.3	68.9	59.1	61.1	95.7	97.1
14 (1)	60.3	24.2	43.3	64.7	97.9	38.1	45.5	67.2	61.6	63.5	99.5	96.3
Blanks 1	11.7	11.6	11.8	12.1	—	—	—	—	—	—	—	—
2	11.1	12.1	11.7	11.9	—	—	—	—	—	—	—	—
3	12.3	11.9	11.9	12.2	—	—	—	—	—	—	—	—
Background	11.5	11.3	11.3	11.7	—	—	—	—	—	—	—	—

Experimental data are given in Fig. 3. In column 1, the numbers in parentheses refer to the leucine residues expected in each peptide. Blanks were obtained from three different areas in each fingerprint.

the computed values of the specific activities of the N-terminal and C-terminal amino acids of both chains are approximately the same after 6 and 9 min incubation of the reticulocytes with ^3H -leucine. Therefore, the rate of incorporation of isotope into the N-terminal residue relative to the C-terminal residues is the same for both chains, indicating no reduction in the rate of assembly or termination of the β chain as a result of the mutation. Other mechanisms by which the amount of gene product could be reduced are a decreased rate of initiation of β^{KW} chain synthesis on the mRNA and a decreased amount of available mRNA as a result of either instability of the mRNA, or a reduced rate of transcription of the abnormal locus. The available data on translation and transcription in eukaryotes do not offer any reason why a change of nucleotide at this position of the structural gene should result in a reduced amount of product. If we speculate, however, and there is a precedent in known tRNAs (ref. 6) that mRNA normally assumes a stable secondary structure dictated by the nucleotide sequence, then there is a mechanism by which initiation of mRNA could be responsible for the low levels of β^{KW} . For example, the nucleotide sequence around the codon for β 132 (H10) lysine could be important in the maintenance of the proposed secondary structure of the mRNA, such that the initiation codon (AUG) was freely available for reaction with the available ribosomal subunits. The nucleotide change responsible for the mutation Lys-Gln, that is, the transversion A-C, could conceivably alter the secondary structure sufficiently to make the initiation codon less available for interaction. By the same argument, the normal codon could also be involved in the maintenance of a stable secondary structure which contributes to the protection of the mRNA during transport from the nucleus. Then the transversion (A-C) could so reduce the stability of configuration that the normal amount of mRNA is prevented from reaching the cytoplasm and the ribosomes.

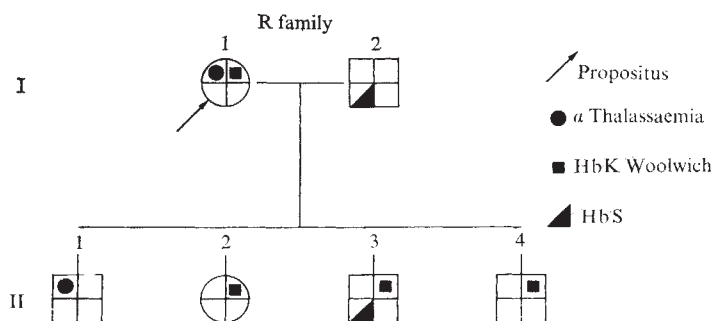
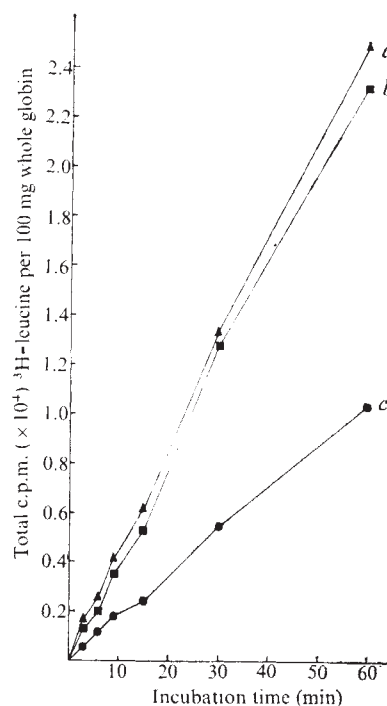
**Fig. 1** Family pedigree of the propositus I-1.

Fig. 2 *In vitro* incorporation of ^3H -leucine into the α , β and β^{KW} chains of the propositus. Reticulocyte-rich red cells prepared from the blood of the propositus¹² were pre-incubated for 15 min at 37°C in the medium of Lingrel and Borsook¹³. $250\ \mu\text{Ci}$ ^3H -leucine ($0.007\ \mu\text{mol}$) were added and samples of cells removed at time intervals from 3–60 min then added to a ten-fold excess of ice cold isotonic saline. The cells were washed several times, lysed and the haemolysate added directly to acid acetone at -20°C . The globin chains from 100 mg whole globin samples were separated on Whatman CM23 cellulose using a linear gradient of Na^+ in 8 M urea¹⁷ pH 6.7. The protein fractions corresponding to the α , β and β^{KW} chains were pooled separately and dialysed against 4×10^{-1} 0.5% formic acid. The total volume of each solution was measured by weight and aliquots were added to 15 ml of scintillator solution (Triton X100, 500 ml; toluene, 1,000 ml; PPO 5 g) (ref. 14) and counted in a 'Tracerlab Coru Matic 200' liquid scintillation spectrometer. a, α chain; b, β^A chain; c, β^{KW} chain.

The propositus I-1 had the additional complication that, besides the signs of a β^+ thalassaemia, the overall imbalance in globin synthesis ($\alpha:\beta$) was the result of a deficiency in α chain production (Table 2). The ratio $\alpha^A/\beta^A+\beta^{KW}$ was in the range associated with silent α thalassaemia. Since the results have shown an absolute decrease in the amount of product from one β locus, however, it seems that she might

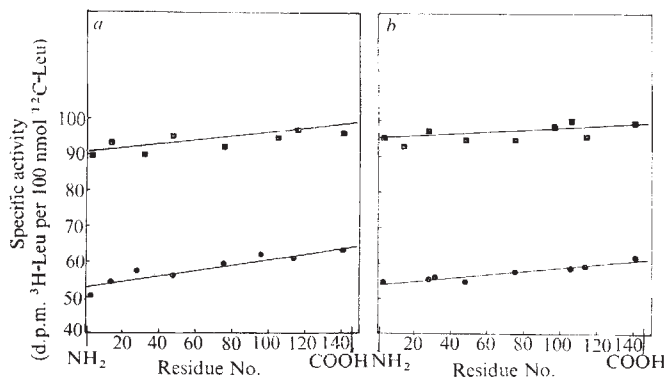


Fig. 3 Comparison of the rates of assembly of the β^A and β^K chains. The gradient of radioactivity in the globin chains after 6 (●) and 9 (■) min incubation with ^3H -leucine. The method used has been described previously¹⁸ a, β^K Woolwich chain; b, β^A chain.

have a compensated α thalassaemia; α thalassaemia, β^{K+} thalassaemia. The haematological data for her son II-1 (Table 1) indicated that he has inherited the α thalassaemia gene but, as yet, he has not been available for *in vitro* biosynthetic studies. On diagnosing α thalassaemia, iron deficiency has to be considered. The serum iron levels in this family were all slightly low with a total serum iron binding capacity in the higher range of normal. As they were all similar, one would not expect this to lower the Hb A₂ in II-1 and not in the others; α thalassaemia in association with β thalassaemia has been described^{10,11}. The results from the *propositus* are similar to those described in one family of Italian origin¹⁰. The amount of Hb KW in the cells of the *propositus* is lower than in either of her children, but it is recognised that an α thalassaemia can reduce the proportion of an associated β chain variant⁴. As the results of the *in vitro* biosynthetic studies do not implicate assembly or release of the completed chains as a possible point of interaction, it is difficult to understand at what other step in biosynthesis a deficiency of α chains could produce this effect, unless at the level of initiation.

Usually (though not invariably) β^A thalassaemia is associated with typical haematological features and these are not seen here. Of the important criteria of thalassaemia, the two found with Hb K Woolwich are the reduction of β -chain synthesis and raised Hb A₂.

A. LANG
H. LEHMANN

Medical Research Council,
Abnormal Haemoglobin Unit,
University Department of Biochemistry,
Addenbrooke's Hospital,
Hills Road, Cambridge CB2 2QR, UK

P. A. KING-LEWIS

Haematology Department,
St George's Hospital,
London SW17 0QT, UK

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Effect of β -N-oxalyl-L- α , β -diaminopropionic acid on glutamate uptake by synaptosomes

THE unusual amino acid β -N-oxalyl-L- α , β -diaminopropionic acid (ODAP), isolated from the seeds of *Lathyrus sativus* is a potent neurotoxin¹⁻³. It produces biochemical changes in the brain typical of an excitant amino acid and is implicated in the aetiology of human neurolathyrism caused by eating the seeds of *L. sativus*⁴⁻⁶. It may act as a glutamate antagonist: ODAP inhibits glutamate oxidation⁷ possibly by inhibiting glutamate uptake in bovine brain mitochondria; it also acts as a competitive inhibitor of glutamate uptake in certain strains of yeast⁸, and a similar process might occur at the synaptic level. Any effect of ODAP on glutamate uptake at synapses is significant in view of the neurotransmitter function of glutamate, which seems to be neuroexcitatory as well as neurotoxic⁹⁻¹². But Balcar and Johnston¹³ have shown with rat brain slices that ODAP does not inhibit the glutamate uptake by the high affinity system.

The high affinity uptake of amino acids such as glutamate, aspartate, glycine and γ -aminobutyric acid by nervous tissue slices and homogenates reflects at least in part their accumulation by the nerve terminals¹⁴⁻¹⁶. We have studied the *in vitro* effect of ODAP on glutamate uptake by synaptosomes isolated from the brain of 12-day-old rats and from monkey spinal cord. We chose these tissues because ODAP produces convulsions in young rats when injected intraperitoneally and precipitates hind leg paralysis in monkeys when introduced into the cerebrospinal fluid through the lumbar route¹⁻³.

Synaptosomes were isolated from the whole brain of rats by the procedure of Eichberg *et al.*¹⁷ except that the P2 pellet was isolated by centrifuging the P₁ supernatant at 7,000g for 30 min instead of at 10,000g for 1 h. This modification by Levitan *et al.*¹⁸ results in a purer preparation of synaptosomes. Synaptosomes were isolated from the monkey spinal cord by the procedure described¹⁹ for the guinea pig spinal cord. Two distinct synaptosomal bands, seen at 1.0-1.2 and 1.2-1.4 M sucrose after density gradient centrifugation in a SW25-1 rotor for 2 h, were collected. These synaptosomal fractions were diluted to 0.4 M and the pellet collected by centrifugation at 15,000g for 20 min. It was used for amino acid uptake studies, conducted essentially according to Peterson and Raghupathy²⁰ (Figs 1-3). The integrity of synaptosomes were ascertained by measuring the lactic dehydrogenase activity of the samples²¹ and demonstrating that very little radioactivity

was retained on the filter when the samples after incubation were lysed with water before filtration.

Synaptosomes isolated from rat brain showed a typical biphasic uptake pattern for glutamate (Fig. 1). The low and high affinity uptake systems were characterised by K_m values of $5 \times 10^{-3} \text{M}$ and $2.5 \times 10^{-3} \text{M}$ respectively. We did not take into account glutamate in the synaptosomal preparation but the K_m values obtained were similar to those reported for glutamate uptake in rat and cat nervous tissues^{14,15}. Addition of ODAP at 10^{-3}M concentration produced around 15–20% inhibition of glutamate uptake by the low as well as high affinity systems. The Lineweaver-Burk analysis of the data indicates a noncompetitive type of interaction (Fig. 1). The pattern remains essentially the same at ODAP concentration of $5 \times 10^{-3} \text{M}$ or 10^{-4}M .

Monkey spinal cord synaptosomes also exhibited a biphasic pattern of glutamate uptake with K_m values of $1.2 \times 10^{-3} \text{M}$ and 0.5×10^{-3} for the high and low affinity uptake systems respectively (Fig. 2). Similar results have been reported for rat and cat spinal cord slices and homogenates^{14,15}. Addition of ODAP to the incubation medium showed a competitive inhibition of glutamate

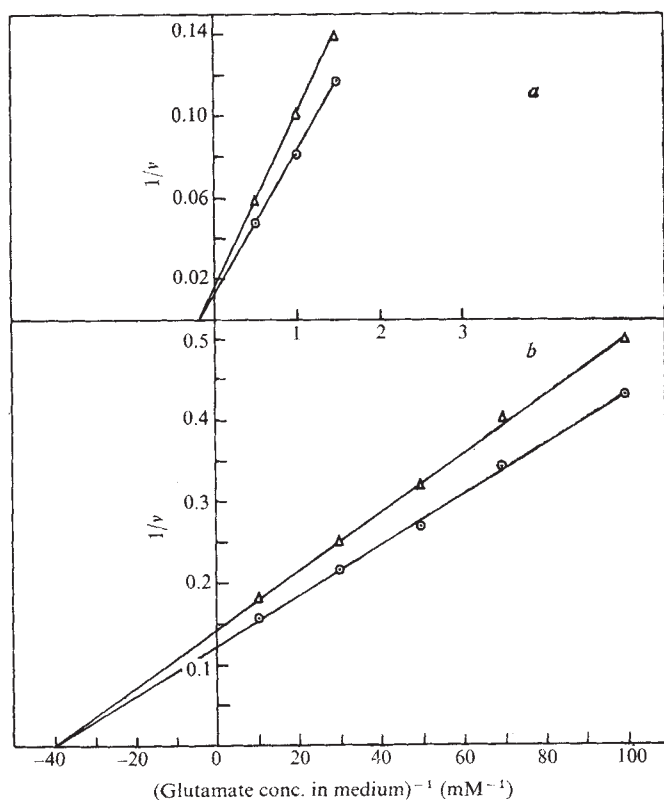


Fig. 1 Effect of ODAP on the uptake of glutamate by young rat brain synaptosomes. Synaptosomes ($200 \mu\text{g}$ protein ml^{-1}) were incubated with $0.25 \mu\text{Ci}$ of $\mu\text{-}^{14}\text{C}$ L-glutamate (99 mCi mmol^{-1}) mixed with unlabelled L-glutamate to give the required concentration in a final volume of 1 ml . Incubation were carried out in 5 ml conical flasks at 37°C for 3 min in a Dubnoff shaker. The medium composition was: 10 mM Tris; 150 mM NaCl; 15 mM MgCl_2 adjusted to pH 7.4 . Reactions were started by the addition of synaptosomes. All incubations were terminated by the addition of 2 ml of ice cold medium followed by quantitative transfer to and washing with 30 volumes of the same buffer over Millipore filters ($0.45 \mu\text{m}$ HAWP). The dried filters were then counted in 10 ml of Cocktail (0.5% PPO (w/v) in Toluene) in a Beckman LS-100 scintillation counter. v is expressed as nmol glutamate taken up per mg protein per 3 min . *a*, Low affinity uptake; *b*, high affinity uptake; $\text{O}-\text{O}$, Control uptake; $\Delta-\Delta$, uptake in the presence of ODAP (10^{-3}M).

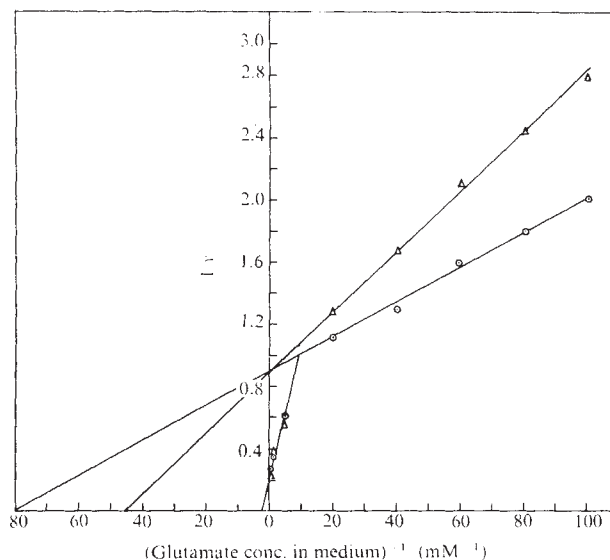


Fig. 2 Effect of ODAP on glutamate uptake by monkey spinal cord synaptosomes. Conditions of incubation as in Fig. 1. $\text{O}-\text{O}$, Control uptake; $\Delta-\Delta$, uptake in the presence of ODAP (10^{-3}M).

uptake by the high affinity uptake system. ODAP has practically no effect on the low affinity uptake of glutamate.

We examined the specificity of ODAP action by studying its effects on glycine uptake, since a high affinity uptake system for this amino acid has been shown to exist only in the spinal cord preparations but not in those of the cerebral cortex of rat and cat¹⁴⁻¹⁶. We found that a biphasic pattern for glycine uptake also exists in the monkey spinal cord synaptosomes, but ODAP has very little effect on either the low or high affinity uptake of glycine (Fig. 3).

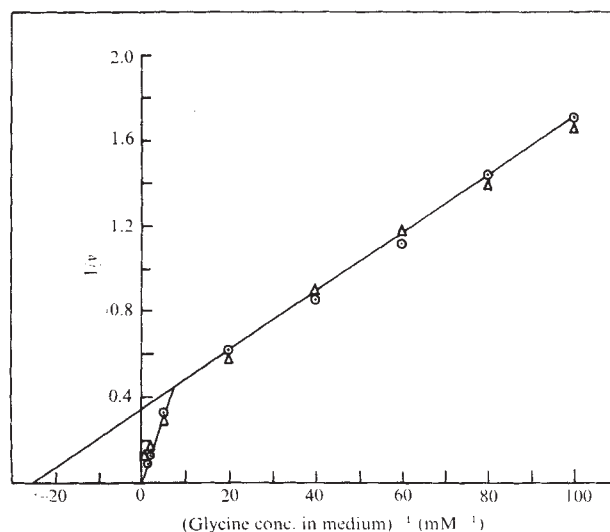


Fig. 3 Effect of ODAP on glycine uptake by monkey spinal cord synaptosomes. Conditions of incubations as for glutamate (Fig. 1) with the following modification. The medium had reduced NaCl (125 mM) but with additional KCl (25 mM). μCi of $2\text{-}^{14}\text{C}$ -glycine (27 mCi mmol^{-1}) was used in combination with unlabelled glycine to give the required concentrations in a final volume of 1 ml . v is expressed as nmol glycine taken up per mg protein per 3 min . $\text{O}-\text{O}$, Control uptake; $\Delta-\Delta$, uptake in the presence of ODAP (10^{-3}M).

ODAP does therefore inhibit glutamate uptake by synaptosomes isolated both from young rat brain and from monkey spinal cord. The lesser potency of ODAP and the type of interaction observed in the rat brain synaptosomes might be a species characteristic or could be due to the presence of a more heterogeneous population of synaptosomes, with affinity uptake systems for transmitter amino acids different from the monkey spinal cord. The localisation of glutamic and aspartic acids in synaptosomes separable from synaptosomes storing other amino acids has been reported²².

The high affinity uptake system of synaptosomes is recognised to be important for the removal of amino acid transmitter from the synaptic environment^{14,15}. Inhibition of this uptake, even the 15–20% inhibitory effect of ODAP seen for the rat brain synaptosomes, could be significant because of the extremely low concentration of glutamate required to cause excitation¹⁰. It is also interesting that ODAP inhibits glutamate uptake but not glycine uptake in monkey spinal cord synaptosomes. Since glycine is an inhibitory transmitter at the spinal cord level, these results could mean that the effects of ODAP in causing convulsions in young rats and hind leg paralysis in monkeys are primarily neuroexcitatory, and are possibly mediated by glutamate. We have observed similar results with regard to the uptake of L-aspartate (unpublished results). Furthermore, ODAP administered *in vivo* to young rats is localised in synaptosomes and can cause the release of excitatory amino acids from synaptosome preparations (manuscript in preparation).

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J. LAKSHMANAN
G. PADMANABAN

Department of Biochemistry,
Indian Institute of Science,
Bangalore 560012, India

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Quasi morphine-abstinence syndrome

ASSUMING that morphine induces dependence in response to continued interaction of the drug with an unidentified biochemical process of neurones, then suitable pharmacological manipulation might produce a paradigm of the morphine abstinence syndrome in experimental animals not exposed to any opiate. If so, the way in which such a quasi-abstinence syndrome was produced might throw light on the endogenous biochemical process involved¹.

In attempting to produce a quasi-abstinence syndrome, we used theophylline, naloxone and 5-hydroxytryptophan (5-HTP). Theophylline was used because it inhibits the phosphodiesterase of rat brain² and would therefore preserve brain cyclic nucleotides and because theophylline or cyclic 3', 5'-adenosine monophosphate (cyclicAMP) intensifies morphine dependence in mice³. Naloxone was used because it has been shown to produce an abstinence-like effect in ileum isolated from naive guinea pigs⁴. 5-HTP was used because the ileum from morphine-treated guinea pigs responded more intensely to the product of its enzymatic decarboxylation, 5-hydroxytryptamine (5-HT), than did ileum from naive animals⁵.

Male albino rats were treated orally with theophylline (100 mg kg⁻¹) in 20% w/v gum acacia or with the vehicle. At either 1 h or 1.75 h later, rats received 0.9% w/v NaCl in water (saline) subcutaneously or intraperitoneally, or 5-HTP (200 mg kg⁻¹) in saline intraperitoneally, according to the treatment schedule. Two hours after the first treatment with theophylline or vehicle, animals were challenged with a subcutaneous injection of either naloxone hydrochloride (10 mg kg⁻¹) in saline; saline; diamorphine hydrochloride (heroin) (1 mg kg⁻¹) in saline; or heroin (1 mg kg⁻¹) + naloxone hydrochloride (0.2 mg kg⁻¹) in saline. For 15 min after challenge the behavioural responses of the rats were recorded by observers who did not know what treatment each animal had received, as previously described⁶. Before each treatment and at the end of the observation period rats were weighed. For comparison, morphine dependence was induced with a single subcutaneous injection of a sustained-release emulsion containing 150 mg kg⁻¹ morphine and abstinence effects were observed 24 h later after subcutaneous challenge with 1 mg kg⁻¹ naloxone⁶. The incidence of responses was expressed as the presence or absence of a response in each animal observed. Weight changes were transformed into percentages and group comparisons were made using the Mann-Whitney U test. Comparable weight changes were not available for rats exposed for 24 h to the sustained-release morphine. For all other responses, probabilities were computed by means of the Fisher's exact probability test for a 2 × 2 contingency table. Weights of drugs are expressed as weights of active base.

Table 1 summarises the responses of rats to the various treatment schedules used. Comparison of responses to theophylline and vehicles with those to vehicles alone shows that theophylline significantly increased the incidence of eight responses — irritability to touch, diarrhoea, ptosis, body shakes, head shakes, rearing, restlessness and weight loss. Naloxone alone increased the incidence of only two of these responses, irritability to touch and weight loss, as compared with controls receiving vehicles; but, after theophylline, naloxone produced a significantly higher incidence of four further responses (compared with theophylline alone), namely jumping, teeth chattering, irritability to handling and paw tremor and increased the significance of rearing and restlessness.

Table 1 Production of abstinence-like responses in rats by theophylline, 5-hydroxytryptophan and naloxone, without exposure to opiate.

Response	% incidence of responses after treatment schedule							
	(a) V+V+V	(b) V+V+N	(c) T+V+V	(d) T+V+N	(e) V+HP+V	(f) T+HP+V	(g) T+HP+N	(h) M+N ¹
Jumping	3	0	4	22 a b c e f	0	0	8	64 a-g
Teeth chattering	8	16	17	28 a b e f	4	4	19	75 a-g
Irritable to touch	3	36 a	35 a	31 a	23 a	50 a e h	58 a d e h	17
Irritable to handling	33	56	50	67 a	65 a	62 a	73 a	69 a
Diarrhoea	25	32	48 a	44	50 a	38	31	89 a-g
Chewing	33	32	52	44	35	46	46	83 a-g
Ptois	33	37 d	61 a d	25	54	54	46	72 a d g
Body shakes	3	0	35 a b e f g	17 b	4	4	8	33 a b e f g
Head shakes	44	64	83 a e f h	75 a e f	42	64	73 a e f	58
Paw tremor	0	0	13	14 a	0	4	4	33 a b c d e f g
Rearing	75 e	84 e	94 a e	100 a e	46	88 e	100 a e	100 a e
Restlessness	44 e	36	71 a b e	83 a b e	19	88 a b e	92 a b e c	74 a b e
Mean % weight loss	0.6	1.6 a	1.9 a	2.3 a	0.8	1.8 a e	1.9 a e	NT
Maximum no. of rats	36	25	46	36	26	26	26	36
Mean total	25.3	32.8	46.9	45.8	28.5	41.8	46.5	63.9

V, Vehicle corresponding with drug; N, naloxone, 10 mg kg⁻¹ subcutaneously (s.c.); N¹, naloxone, 1 mg kg⁻¹ s.c.;

T, theophylline, 100 mg kg⁻¹ orally; HP, 5-hydroxytryptophan (5-HTP) 200 mg kg⁻¹ intraperitoneally;

M, morphine, 150 mg kg⁻¹ s.c. in a sustained-release preparation; NT, not tested.

After pretreatment with theophylline, or vehicle (2 h), saline (1 h) or 5-HTP or vehicle 15 min previously, rats were challenged with vehicle or naloxone. After pretreatment with morphine 24 h previously, rats were challenged with naloxone. Responses were recorded during 15 min after challenge. Letters a-h in the body of the Table indicate comparisons between columns where a significantly greater effect was found; where the letter is italic, $P < 0.01$, where it is not, $P < 0.05$.

5-HTP and vehicles, compared with vehicles alone, produced an increased incidence of three responses — irritability to touch, irritability to handling and diarrhoea, but reduced the incidence of rearing and restlessness. Theophylline, 5-HTP and vehicle gave a significantly increased incidence of irritability to touch, rearing, restlessness and weight loss compared with 5-HTP and vehicles. Treatment with theophylline, 5-HTP and naloxone gave a significantly increased incidence of six of the thirteen responses recorded compared with vehicles alone.

Table 1 also gives the abstinence effects observed in a similar way after withdrawal of morphine, precipitated with 1 mg kg⁻¹ naloxone, 24 h after administration to rats of a sustained-release preparation of morphine. The incidence of many of the responses is still higher than after theophylline, 5-hydroxytryptophan and/or naloxone.

Table 2 Blockade by heroin of behavioural responses of rats to theophylline and removal of blockade by naloxone.

Response	% incidence of responses after treatment schedule		
	a T+V+V ¹	b T+V+H	c T+V+(H+N)
Jumping	0	10	20
Teeth chattering	0	0	10
Irritable to touch	50 b	0	50 b
Irritable to handling	70 b	10	70 b
Diarrhoea	80 b	10	50
Chewing	70 b	0	30
Ptois	70 b	20	40
Body shakes	10	0	10
Head shakes	90 b	0	90 b
Paw tremor	10	0	0
Rearing	90 b	20	100 b
Restlessness	90 b	0	100 b
Mean % weight loss	1.8	1.5	1.6
Maximum no. of rats	10	10	10
Mean total	52.5	5.8	47.5

T, theophylline, 100 mg kg⁻¹ given orally at 0 h; V, saline, 10 ml kg⁻¹ given subcutaneously (s.c.) at 1 h; V¹, saline s.c. H, heroin, 1.0 mg kg⁻¹ s.c. or H + N, heroin 1 mg kg⁻¹ + naloxone, 0.2 mg kg⁻¹ s.c. at 2 h. Other details as in Table 1.

Table 2 summarises experiments in which heroin (1 mg kg⁻¹) was tested as an inhibitor of responses induced by theophylline. Heroin largely suppressed the quasi-abstinence effects. Nal-

oxone (0.2 mg kg⁻¹) was found to overcome this inhibition by heroin.

Of the various treatment schedules, theophylline with naloxone produced the closest resemblance to a true morphine abstinence syndrome (Table 1); but the presence of theophylline in any schedule led to a high incidence of several quasi-abstinence responses. 5-HTP produced some behavioural responses resembling those of abstinence, but these were fewer than with theophylline. The results of treating naive rats with theophylline resembled in four ways the abstinence syndrome during morphine withdrawal without precipitation by an antagonist: first, many of the same responses increased in incidence; second, naloxone increased the incidence of responses in both conditions; third, heroin lessened the incidence of responses; and fourth, naloxone antagonised this effect of heroin. We therefore apply the term 'quasi-abstinence syndrome' to the behavioural state produced in the naive rat by theophylline.

The ability of theophylline to produce quasi-abstinence effects indicates that cyclic nucleotides may be involved in morphine dependence. This accords with the findings that cyclic AMP or theophylline antagonised the antinociceptive effect of morphine in the rat^{7,8} or mouse⁸ and enhanced tolerance and dependence in the mouse³, although other cyclic nucleotides were inactive. This observation is consistent with the suggestion⁹ that the raised level of protein kinase found in rat brain after morphine withdrawal may contribute directly to the signs of abstinence.

The contribution of 5-HTP to the quasi-abstinence syndrome reinforces a comment made some years ago¹⁰. Also, it is consistent with the finding that the ileum isolated from morphine-tolerant guinea pig is more responsive to 5-HT than is normal ileum⁵. That 5-HT inhibits noradrenaline stimulation of adenylyl cyclase¹¹, makes plausible an association between this effect of 5-HT and a cyclic AMP mechanism.

In vivo studies alone cannot determine the precise mechanism of action of a drug, but they can indicate where this mechanism should be sought. The present studies have indeed pointed to a further exploration of the interaction of morphine with cyclic nucleotides; and it has been reported that morphine potentially antagonises the activation by PGE₁ or PGE₂ of cyclic AMP formation by rat brain or intestine homogenate¹². It therefore seems possible that morphine tolerance and dependence may arise through compensatory changes in processes related to this PGE – cyclic AMP interaction.

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H. O. J. COLLIER
D. L. FRANCIS
G. HENDERSON*
C. SCHNEIDER

Miles Laboratories Ltd.,
Stoke Poges, Slough SL2 4LY,
Buckinghamshire, UK

*Present address: Unit for Research on Addictive Drugs, Marischal College, University of Aberdeen, Scotland, AB9 1AS.

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Neurotrophic control of colchicine effects on muscle?

COLCHICINE, a substance known to block axonal transport^{1,2}, has been reported to mimic the effects of denervation on mammalian skeletal muscles³⁻⁵. These effects, which include an increased extrajunctional sensitivity to acetylcholine (Ach) and a fall in resting membrane potential, occur a few days after colchicine is applied locally to the nerve in doses that do not seem to interfere with nerve impulse conduction, neuromuscular transmission, or muscle tension evoked by stimulation of the nerve³⁻⁵. It seems, therefore, that denervation-like changes may be induced in the presence of normal muscle activity and this suggestion is supported by experiments reported here in which the diaphragms of rats with apparently normal respiration became supersensitive to Ach after injection of colchicine into one of the legs (mean extrajunctional Ach sensitivity of 22 fibres from three rats was 4.6 mV nC⁻¹, range 0-30 mV nC⁻¹). It has been suggested that colchicine produces these changes in muscle by blocking some component of axonal transport, thus interfering with the neurotrophic control of muscle^{3,4}. Colchicine also has a systemic effect⁵, however, and its effect on muscle might be direct and occur independently of any of its effects on nerve. Here I examine this possibility.

The experiments were performed on young white rats each weighing about 200 g. The sciatic nerve was resected on one side, and two platinum stimulating electrodes were implanted in the denervated leg, one on each side of the soleus muscle. After waiting 1 d for neuromuscular transmission to fail and degeneration of motor nerve terminals to begin⁶, the muscle was stimulated directly by intermittent trains of stimuli at 10 Hz until the acute experiment. Such stimulation largely prevents the development of Ach supersensitivity in denervated muscle⁷. Because colchicine is reported to have its greatest effect on muscle after 4 d (ref. 4), it was injected (3.5 µl of a 0.2 M solution) intramuscularly high in the thigh 4 d before the acute

experiment. Fourteen days after denervation, the soleus muscles were removed from both sides and placed in a chamber superfused with oxygenated rat Ringer's solution at room temperature. Both the resting membrane potential and the sensitivity to electrophoretically applied Ach were examined in single muscle fibres by conventional techniques⁸. Control animals were similarly treated but received no colchicine; in some of these controls, the unstimulated soleus muscle was also denervated.

Four days after the injection of colchicine, the mean extrajunctional Ach sensitivity of fibres with intact innervation (61 fibres, six muscles) was 3.2 mV nC⁻¹ (range 0.2-14.3 mV nC⁻¹) (Fig. 1a, open columns). A similar sensitivity and distribution

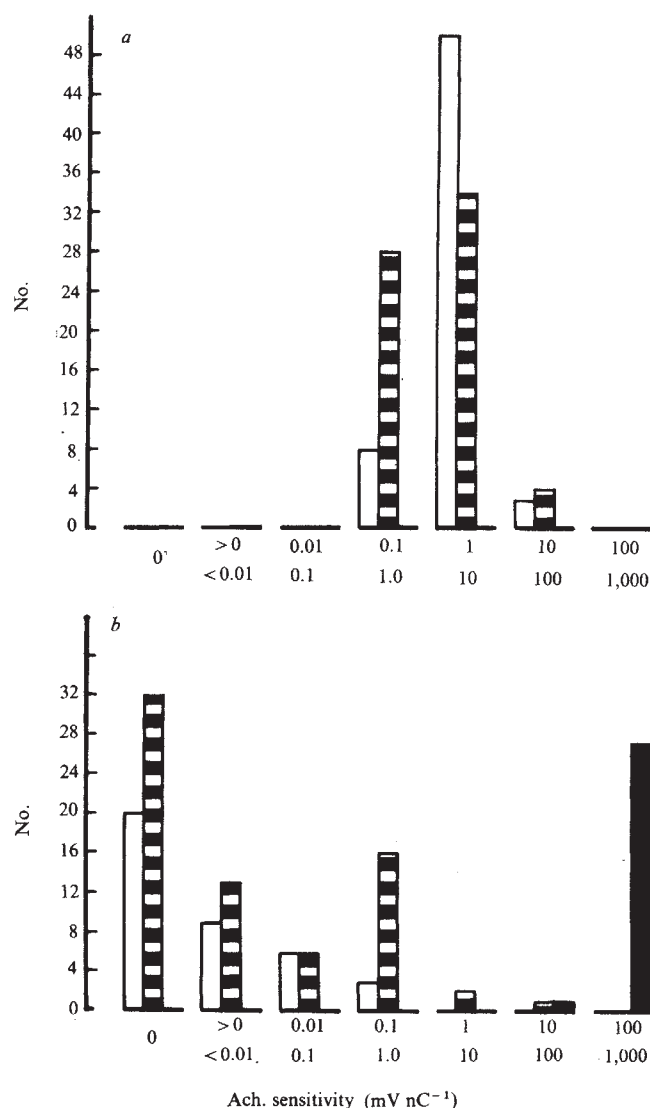


Fig. 1 Extrajunctional Ach sensitivity of soleus fibres from innervated (open columns), denervated stimulated (striped columns) and denervated muscles (solid columns). Denervation lasted 14 d. Stimulation took place from day 2 to day 14 after denervation. Ach sensitivity was measured *in vitro* at 2 mm from the myotendinous junction and is expressed as mV depolarisation per nC charge passed through a micropipette containing 3 M AchCl. Because the supersensitivity following either denervation or colchicine treatment is approximately equal along the membrane lying between the endplate and the myotendinous junction^{8,9}, these values may be taken as representative of the average extrajunctional sensitivity for the whole fibre. a, 270 µg Colchicine injected intramuscularly high in the thigh on day 10, that is 4 d before the Ach-sensitivity was tested; b, no colchicine was given.

about the mean (3.2 mV nC^{-1} , range $0.1\text{--}15.0 \text{ mV nC}^{-1}$, excluding one fibre with 57 mV nC^{-1}) was found in denervated stimulated soleus fibres (66 fibres) from the opposite leg of the same animals (Fig. 1a, striped columns). In the absence of colchicine the same stimulation largely blocked the supersensitivity of denervated fibres (70 fibres, five muscles) so the majority of these were insensitive or only very weakly sensitive (Fig. 1b, striped columns) and were indistinguishable from normal fibres (Fig. 1b, open columns). In two of the five denervated stimulated muscles, a few fibres had a higher sensitivity than normal. These fibres generally occurred in small groups clearly separated from the insensitive or weakly sensitive fibres in the same muscle. Their high sensitivity may, therefore, be caused by inadequate stimulation or local injurious effects of the stimulation⁷. In contrast, denervated but unstimulated fibres (27 fibres, three muscles) were all highly sensitive with a mean sensitivity of 250 mV nC^{-1} (range $50\text{--}525$) (Fig. 1b, solid columns). It seems that colchicine raises the Ach sensitivity of both innervated and denervated stimulated fibres to about the same extent.

Colchicine had a comparable effect on resting membrane potential. Both innervated and denervated stimulated soleus fibres exposed to colchicine had similar relatively low mean resting membrane potentials; $67.4 \pm 4.8 \text{ mV}$ (mean $\pm$ s.d.) and $67.5 \pm 4.2 \text{ mV}$ (mean $\pm$ s.d.), respectively. In the absence of colchicine the corresponding values were higher: $70.9 \pm 4.4 \text{ mV}$ and $74.1 \pm 4.9 \text{ mV}$. The lowest membrane potentials were recorded in fibres that were only denervated ($61.6 \pm 5.3 \text{ mV}$). The effect of colchicine on both resting membrane potential and Ach sensitivity was thus similar to that of denervation but less pronounced.

All innervated muscles exposed to colchicine twitched vigorously to stimulation of the nerve and miniature endplate potentials of approximately normal amplitude and frequency were observed in all fibres examined. In denervated stimulated muscles, there was no twitch to nerve stimulation, and no miniature endplate potentials were observed.

The increase in extrajunctional Ach sensitivity and fall in resting membrane potential induced by colchicine in innervated rat soleus muscles confirm other findings³⁻⁵. In addition my experiments show that similar changes developed in denervated muscles that were kept active by direct stimulation. As direct stimulation in the absence of colchicine has been shown to prevent such changes⁷ it seems that colchicine has some effects on the muscle membrane that are not mediated by the nerve. These effects of colchicine on innervated muscle should therefore not be taken as evidence for the neurotrophic control of extrajunctional membrane properties. The mechanism behind the effect of colchicine on muscle is not known, and is in itself of some interest.

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TERJE LOMO

*Institute of Neurophysiology,
University of Oslo,
Karl Johansgt, 47,
Oslo 1, Norway*

Pseudorotation mechanism of ATP hydrolysis in muscle contraction

AN analysis of the reaction mechanism of ATP hydrolysis may elucidate how ATP provides energy for muscle contraction. Kinetic studies of the mechanism of ATP hydrolysis catalysed by myosin and its proteolytic subfragments^{1,2}, revealed at least two intermediates before the rate limiting step: (1) enzyme-bound ATP (M^*ATP) and (2) an intermediate ($M^*ADP \cdot P_i$) that yields ADP and P_i when a quenching reagent such as $HClO_4$ is added. Trentham *et al.*² proposed that the rate limiting step is the transition from $M^*ADP \cdot P_i$ to the Michaelis product complex $M \cdot ADP \cdot P_i$. That $M^*ADP \cdot P_i$ is distinct from the Michaelis product complex is supported by observations made using techniques which include spin labelling³, difference ultraviolet absorption spectroscopy⁴, extrinsic probe fluorescence⁵ and intrinsic myosin fluorescence⁶. Each method shows that the conformation of myosin during ATP hydrolysis differs from the conformation induced by the binding of ADP and P_i . This conclusion is supported further by the observation that the free energy change for the reversible reaction $M^*ATP \rightleftharpoons M^*ADP \cdot P_i$ is only -1.3 kcalories per mol (ref. 7). Although comparable in energy with M^*ATP , $M^*ADP \cdot P_i$ nevertheless seems to be closely related to products because it decays to ADP and P_i when exposed to a quenching reagent. Although this decay to ADP and P_i has been widely interpreted as indicating that ATP is already cleaved in $M^*ADP \cdot P_i$, it could alternatively be interpreted in terms of an uncleaved but labile reaction intermediate.

In the intermediate $P_i \rightleftharpoons H_2O$ exchange⁸ ^{18}O must be incorporated into either M^*ATP or $M^*ADP \cdot P_i$. Sartorelli *et al.*⁹ have shown that when myosin-catalysed ATP hydrolysis is quenched by $HClO_4$ containing $H_2^{18}O$, no ^{18}O is detected either in the P_i cleaved from ATP or in the myosin. This implies that H_2O must enter both the hydrolysis and the intermediate exchange reactions before the formation of $M^*ADP \cdot P_i$. Thus $M^*ADP \cdot P_i$ cannot be some form of anhydride such as a phosphorylated myosin or metaphosphate.

We show here that a pseudorotation reaction mechanism for ATP hydrolysis can account for both the intermediate and medium¹⁰ $P_i \rightleftharpoons H_2O$ exchanges as well as the characteristics of $M^*ADP \cdot P_i$. A pseudorotation mechanism has previously been proposed for ATP synthesis in mitochondrial oxidative phosphorylation¹¹⁻¹³. We have recently shown it to be the only mechanism proposed so far that accounts adequately for the mitochondrial oxygen exchanges¹⁴.

Figure 1 presents a schematic representation of the pseudorotation mechanism of ATP hydrolysis. The pseudorotation process transforms the P-O bond cleaved in hydrolysis from a relatively short, strong and nonreactive equatorial bond (Fig. 1a) to a relatively long, weak and reactive apical bond (Fig. 1b). In Fig. 1a the pair of oxoniums bound by H bonds to the enzyme and apical bonds to the phosphorus centre constitute two entry points for H_2O oxygen. The diagram shows that by dynamic reversal of the H_2O binding step before pseudorotation, the two oxygens in the pair of oxoniums could equilibrate with the H_2O oxygens of the medium. This equilibration could introduce up to two labelled oxygens into the P_i cleaved in ATP hydrolysis.

Under certain conditions, however, all four of the oxygens in the P_i cleaved in ATP hydrolysis can be labelled^{14,16}. Since the enzyme-bound H_2O exchanges with the medium H_2O , some of the time a H_2O molecule must be missing from the active site. In the absence of H_2O the γ -phosphoryl of ATP might undergo a rotation about the bridge P-O bond in a time comparable with the H_2O exchange time. In this way all four of the oxygens in the P_i cleaved from ATP could be labelled with ^{18}O . It must be emphasised that such a rotation could occur only in the absence of the H_2O molecule. In the pentacoordinate intermediate A the γ -phosphoryl would presumably

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be bound so tightly that no rotation could occur. One can also conceive of a mechanism in which complete labelling of P_i occurs into a tightly bound γ -phosphoryl of ATP. For such a mechanism the exchange illustrated in Fig. 1 would be only a prototype.

Since H_2O must enter both the hydrolysis and the intermediate exchange reactions before the formation of $M^*ADP \cdot P_i$, the only alternative to intermediate exchange occurring into M^*ATP seems to be the following sequence of events: (1) cleavage of the P-O bond, (2) rotation of P_i in the active site, (3) spontaneous reformation of the P-O bond, and (4) exchange of enzyme-bound H_2O with medium H_2O . If all four of the oxygens of the cleaved P_i were able to interchange their positions in the active site, then all four of the P_i oxygens could be labelled by this mechanism. However, the weak binding implicit in the rotation of P_i in the active site seems to be inconsistent with the evidence that $M^*ADP \cdot P_i$ is not a Michaelis product complex but rather is energetically comparable with M^*ATP . This alternative therefore seems unlikely.

Thus it seems that exchange occurs directly into the γ -phosphoryl of M^*ATP . Excluding an unlikely rotation of the $ATP \cdot H_2O$ adduct in the active site, direct exchange into M^*ATP implies at least two entry points for H_2O oxygen. This exceedingly restrictive condition would exclude all mechanisms not of the type depicted in Fig. 1.

Concerning the medium exchange, there is a nucleotide requirement as a cofactor that can be satisfied by nucleotide mono-, di- and triphosphates as well as the ATP analogue adenylyl-methylenediphosphonate which does not undergo hydrolysis¹⁶. This implies that the medium exchange does not occur through the reversal of hydrolysis. Medium exchange must therefore occur directly into P_i which is analogous to the requirement that intermediate exchange must occur directly into the γ -phosphoryl of ATP by means of two or more entry points of H_2O oxygen. Thus consideration of the medium exchange independently leads to the same conclusion as that reached from consideration of the intermediate exchange.

A medium exchange separate from the reversal of hydrolysis is similar to the mitochondrial $P_i \rightleftharpoons H_2O$ exchange separate from the reversal of phosphorylation¹⁴. Since the characteristics of this mitochondrial $P_i \rightleftharpoons H_2O$ exchange can be rationalised by a mechanism stereochemically analogous to the mechanism proposed for the mitochondrial $ATP \rightleftharpoons H_2O$ exchange, we propose that the medium exchange can similarly be rationalised by a mechanism stereochemically analogous to the intermediate $P_i \rightleftharpoons H_2O$ exchange.

Equating intermediate *b* in Fig. 1 with $M^*ADP \cdot P_i$ accounts for: (1) the difference between $M^*ADP \cdot P_i$ and the Michaelis product complex, (2) the 'high energy' nature of $M^*ADP \cdot P_i$, (3) the breakdown of $M^*ADP \cdot P_i$ to yield ADP and P_i when a quenching reagent is added, and (4) the breakdown of $M^*ADP \cdot P_i$ without incorporation of ^{18}O into either P_i or myosin.

The mechanism proposed here for the intermediate $P_i \rightleftharpoons H_2O$ exchange is identical to that proposed for the mitochondrial $ATP \rightleftharpoons H_2O$ exchange¹¹ except that the ^{18}O appears in the P_i cleaved from ATP rather than in ATP itself. However, no myosin-catalysed $ATP \rightleftharpoons H_2O$ exchange has been detected^{8,9}. The failure to detect a myosin $ATP \rightleftharpoons H_2O$ exchange is consistent with the low value for the dissociation rate of unhydrolysed ATP ($\leq 0.02 \text{ s}^{-1}$)^{7,17} together with the small steady state concentration of the dissociating species M^*ATP relative to $M^*ADP \cdot P_i$.

Available evidence strongly supports a pseudorotation mechanism of ATP hydrolysis. It is therefore interesting to inquire whether a rationale might be discernible for this mechanism in muscle contraction.

In the model of Lynn and Taylor¹⁸ actin and myosin are not associated in the transition from M^*ATP to $M^*ADP \cdot P_i$. This would require that this transition be an approximately energy conserving process, as recently confirmed by Bagshaw and Trentham⁷. As developed elsewhere with regard to ATP

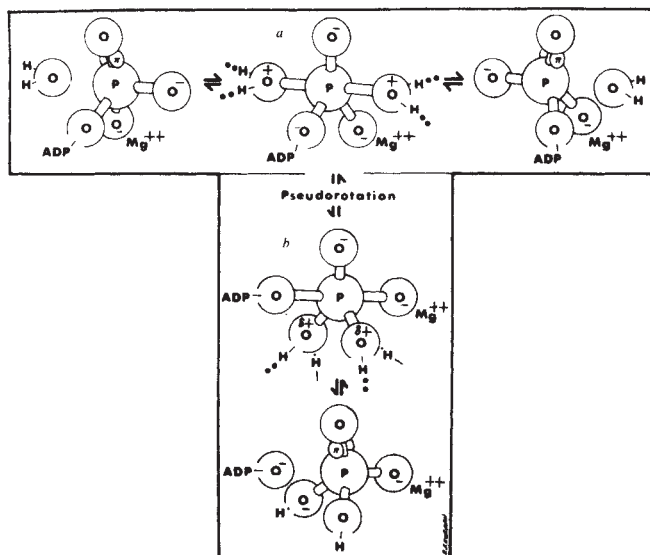


Fig. 1 Pseudorotation reaction mechanism of ATP hydrolysis. Reaction intermediate *a* with ADPO- equatorial has two apical oxoniums. Reaction intermediate *b* depicts ADPO- apical as required for cleavage from the phosphorus centre.

synthesis in oxidative phosphorylation, a fundamental aspect of the pseudorotation mechanism is the pair of oxoniums^{11,12}. Its effect is to stabilise intermediate *a* in Fig. 1 relative to intermediate *b*, thereby providing approximate energy parity between the intermediate resembling reactants and the intermediate resembling products. Thus the pair of oxoniums responsible for a straightforward explanation of the oxygen exchanges could also account for the approximate energy parity between these two intermediates. This suggests a mechanistic basis for the correlation between intermediate exchange activity and muscle contraction noted by Yount and Koshland¹⁵.

In the sliding filament model tension is generated by a change in orientation of the myosin head with respect to the actin filament¹⁹. This implies that actin must initially associate with myosin to give a state other than the thermodynamically most stable state of the actomyosin complex. It seems that actin associates initially with myosin in the $M^*ADP \cdot P_i$ state^{18,20}. As previously noted, there is considerable evidence that the conformation of myosin in the $M^*ADP \cdot P_i$ state differs from that in the Michaelis product complex $M \cdot ADP \cdot P_i$. Thus these two conformations of myosin might be correlated tentatively with the two orientations of the actomyosin cross bridge invoked by Huxley and Simmonds^{21,22} to explain the viscoelastic properties of active muscle fibres. This correlation would also be consistent with the requirement that, because the velocity of muscle contraction decreases with increasing load, the rate limiting step of ATP hydrolysis must be sensitive to tension. Exergonic bond cleavage would therefore occur in the mechanochemically coupled step since in this reaction mechanism cleavage occurs in the transition from $M^*ADP \cdot P_i$ to $M \cdot ADP \cdot P_i$.

These considerations together with the dissociation of actomyosin induced by ATP binding¹⁸ and the refractory state of myosin²⁰ indicate that the contraction cycle involves a sequence of discrete conformational states of myosin. A sequence of discrete conformational states of myosin as an ATPase enzyme requires a sequence of discrete reaction intermediates in ATP hydrolysis. The intrinsically step-wise character of the pseudorotation mechanism is well suited for providing such a sequence of reaction intermediates with appropriate energetic characteristics.

In view of the apparent similarity between the ATP reaction mechanisms in muscle contraction and oxidative phosphorylation, it is of interest to note that the myosin fragment S-1

appears to be evolutionarily related to the mitochondrial coupling factor F_1 (ref. 23).

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JOHN H. YOUNG
JEROME McLICK
EPHRAIM F. KORMAN

School of Pharmacy,
Theoretical Chemistry Institute,
Department of Chemistry, and
Department of the History of Science,
University of Wisconsin, Madison, Wisconsin 53706.

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In vivo enhancement of tyrosine hydroxylation in rat striatum by tetrahydrobiopterin

THE hydroxylation of tyrosine, the rate-limiting step in the biosynthesis of catecholamines *in vivo*¹, depends on various factors: the concentration of apoenzyme and of cofactor (5,6,7,8-tetrahydrobiopterin, BH_4), the availability of molecular oxygen²⁻⁴ and the activity of the system reducing the oxidised pteridine (dihydropteridine reductase⁵). Calculations based on the rate of tyrosine hydroxylation *in vitro* and on the cofactor concentration in brain suggested that the latter might be limiting^{6,7}, although its concentration at the enzyme site is not known. Similar conclusions were reached in experiments with cultures of sympathetic chain of chicks in which addition of biopterin increased the catecholamine content⁸. But no evidence exists for a possible role of BH_4 as limiting factor of tyrosine hydroxylation *in vivo*. We describe here the *in vivo* effect of BH_4 , the natural cofactor of tyrosine hydroxylase⁹, on dopamine formation in rat striatum.

Male albino rats of Wistar origin (Füllinsdorf), 180-200 g,

were injected with an aqueous solution of BH_4 into one lateral brain ventricle (30-1,000 μ g in 10 μ l through a chronically implanted cannula¹⁰) or intravenously (125 mg kg^{-1}). The solutions contained 1% ascorbic acid. At various time intervals after administration of BH_4 , 0.18 μ g L-2, 3-³H-tyrosine in 10 μ l (specific activity) 10 Ci $mmol^{-1}$) were injected intraventricularly. Animals injected with ³H-tyrosine after ascorbic acid served as controls. The rats were decapitated 15 min after ³H-tyrosine injection, the corpora striata immediately dissected and homogenised in 0.4 M ice-cold perchloric acid (1:5 w/v) containing 0.3% ascorbic acid and 10 μ g dopamine. The radioactive catechols were absorbed on alumina¹¹, eluted with 4 ml 0.3 N acetic acid and measured in a liquid scintillation counter. In some experiments the composition of the eluate was checked by paper chromatography (Whatman P81; isopropanol, 0.2 N ammonium acetate, pH 6.0, 1:2; 15 h). Dopamine was the main component besides traces of 3,4-dihydroxyphenylacetic acid. The values were corrected for recovery ($75 \pm 2.3\%$).

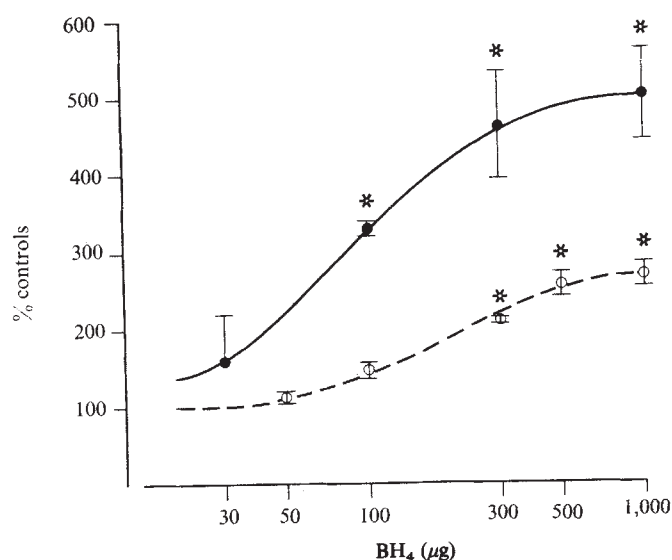


Fig. 1 Concentration of ●, tetrahydrobiopterin (BH_4), and ○, ³H-catechols in the rat striatum following various doses of BH_4 . BH_4 was administered into one lateral brain ventricle alone or immediately before 0.18 μ g ³H-tyrosine intraventricularly. Rats were killed 15 min after injection(s). The figures represent averages with s.e.m. of values from 12-36 determinations. Each determination was carried out with the corpora striata of one rat. Absolute control values: BH_4 $24.5 \pm 3.5 nmol g^{-1}$; ³H-catechols $6.4 \pm 0.8 pmol g^{-1}$. Significance against corresponding controls: * $P < 0.01$; all other values $P > 0.05$.

For the measurement of the cofactor concentrations another group of rats received BH_4 (in 1% aqueous ascorbic acid) intraventricularly (i.v.) (see above) at various time intervals before killing. Animals injected with ascorbic acid alone served as controls. The corpora striata from one rat were homogenised in 0.2 M Na-phosphate buffer, pH 6.5 (1:5 w/v) containing 0.02 M ascorbic acid, heated for 90 s to 100° C and centrifuged. A 10 μ l aliquot of the supernatant was incubated for 60 min at 30° C with: 0.2 M phosphate-citrate buffer, pH 6.4, 20 μ l; 10 mM ascorbic acid, 20 μ l; 0.5 mM L-³H-phenylalanine (SA 40 mCi $mmol^{-1}$), 20 μ l; 70 μ g liver phenylalanine hydroxylase (purified according to Kaufman and Fisher¹², first two steps), dissolved in 20 μ l 0.2 M Na-phosphate buffer (see above); water to a total volume of 130 μ l¹³. Supernatant containing various concentrations of BH_4 (20-100 pmol in phosphate buffer) or phosphate buffer served as standards or blanks, respectively. The reaction was stopped by cooling to 0° C. After addition of 0.5 ml ice-cold Na-acetate buffer (0.2 M, pH 5.5), iodination

was performed using 1 mg N-iodosuccinimide⁷ in 10 μ l dimethylsulphoxide. After 5 min the samples were supplemented with 100 μ l 30% trichloroacetic acid, centrifuged and passed through columns of charcoal (Norit S $\times$ 35)/Dowex 50W $\times$ 4 (10/700 mg) (ref. 7). The effluent (combined with the washing, 1 ml water) containing tritiated water, was measured by liquid scintillation counting. In such a system the amount of tritiated water is proportional to the initial concentration of BH₄ in the incubation medium. In all experiments quenching was corrected by means of internal standards.

Intraventricular administration of increasing amounts (30–1,000 μ g) of BH₄ alone and of BH₄ immediately before ³H-tyrosine caused in the striatum a dose-dependent

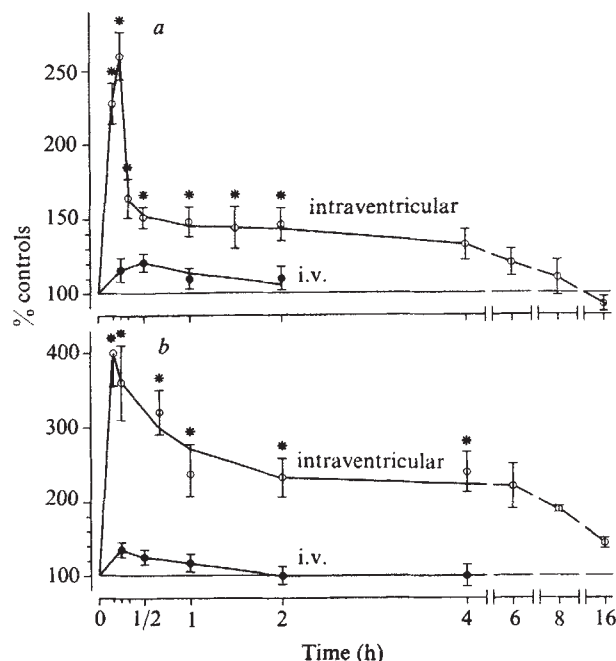


Fig. 2 Time course of *a*, ³H-catechols and *b*, tetrahydrobiopterin (BH₄) in the striatum. BH₄ was administered into one lateral ventricle of the rat brain (0.5 mg per rat) or intravenously (125 mg kg⁻¹) alone or at various time intervals before intraventricular injection of 0.18 μ g ³H-tyrosine injected 15 min before killing. Abscissa: time between injection of BH₄ and decapitation. The figures represent means with s.e.m. of values from 12–36 determinations. Each determination was carried out with the corpora striata of one rat. Absolute control values: BH₄ 29.0 $\pm$ 5.1 nmol g⁻¹; ³H-catechols 7.5 $\pm$ 0.8 pmol g⁻¹. Significance against corresponding controls: **P* < 0.01; all other values *P* > 0.05.

accumulation of the cofactor and an increased accumulation of labelled catechols, respectively (Fig. 1). The time course of accumulation of striatal BH₄ and increased accumulation of ³H-catechols was similar. Maximal values were found 10 and 15 min respectively, after injection of the cofactor. After 4 h the ³H-catechols had almost reached control levels again, whereas BH₄ was still somewhat increased at 16 h (Fig. 2), possibly due to a continuous recycling of the oxidised cofactor.

Intravenous administration of a large dose (125 mg kg⁻¹) of BH₄ alone or immediately before intraventricular injection of ³H-tyrosine caused a small increase of the cofactor and of the ³H-catechol content in the striatum respectively, which did not, however, reach a significant level (Fig. 2). This small effect may be due to poor penetration of the cofactor into the brain.

The enhanced accumulation of labelled catechols in the striatum which paralleled the increased BH₄ content was probably the consequence of an increased synthesis of the amines from ³H-tyrosine. Thus, 250 mg kg⁻¹ α -methyl-*p*-

tyrosine (an inhibitor of tyrosine hydroxylase) i.p. 1 h before ³H-tyrosine diminished both the formation of labelled catechols and their enhanced accumulation due to BH₄ (Fig. 3). An impaired elimination of the labelled catechols from the brain is unlikely since in preliminary experiments BH₄ did not enhance the accumulation of ³H-catechols in the striatum of animals administered L-2,5,6-³H-dihydroxyphenylalanine intraventricularly. This result also confirms that BH₄ acts at the level of the hydroxylation of tyrosine.

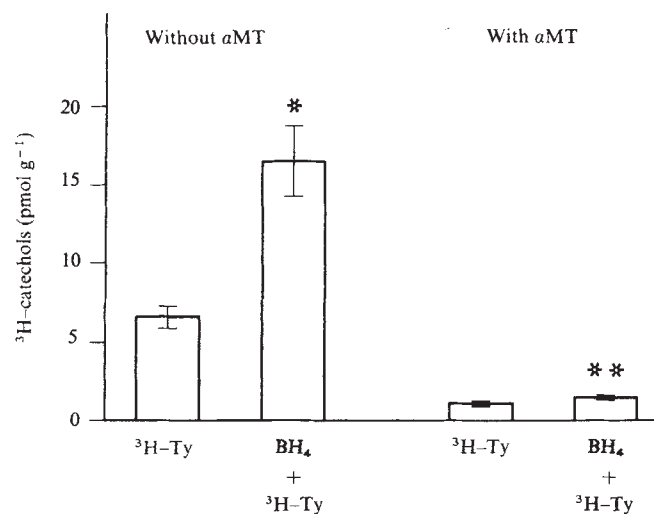


Fig. 3 Effect of α -methyl-*p*-tyrosine (α MT) on the increase of ³H-catechols in rat striatum induced by ³H-tyrosine (³H-Ty) alone or in combination with tetrahydrobiopterin (BH₄). BH₄ (0.5 mg per rat) was injected into one lateral brain ventricle immediately before intraventricular administration of 0.18 μ g ³H-Ty. α MT (250 mg kg⁻¹) was administered i.p. 1 h before ³H-Ty. Rats were killed 15 min after ³H-Ty. The figures represent averages with s.e.m. of 4–6 determinations, each carried out with the corpora striata of one rat. Significance compared with corresponding values with ³H-Ty alone: **P* < 0.01; ***P* > 0.1.

The striatal content of endogenous dopamine and its major metabolite homovanillic acid (both measured fluorimetrically^{11,14}) was not significantly changed 15 min after intraventricular injection of 500 μ g BH₄ (dopamine: controls 8.7 $\pm$ 0.4, BH₄-treated 9.1 $\pm$ 0.2 μ g g⁻¹, *P* > 0.05; homovanillic acid: controls 0.84 $\pm$ 0.07, BH₄-treated 0.80 $\pm$ 0.08 μ g g⁻¹, *P* > 0.05; 12 animals per group). These findings indicate that of the total dopamine stored the newly formed amine is only a minor part which may have entered into the small (functional) pool previously claimed to be preferentially filled with the newly synthesised catecholamines¹⁵.

From these results it can be concluded that in the striatum the amount of the apoenzyme of tyrosine hydroxylase is probably not the limiting factor for the hydroxylation of tyrosine *in vivo*. Under physiological conditions, tyrosine hydroxylase apparently does not work to its full capacity due to the limited concentration of BH₄. It is therefore possible that rapid changes in activity of tyrosine hydroxylase may be connected with modifications in the availability of reduced pteridine cofactor at the enzyme site.

The BH₄ was synthesised by Dr K. J. M. Andrews, Roche Products Limited, Welwyn Garden City, UK.

R. KETTLER
G. BARTHOLINI
A. PLETSCHER

Research Department, Hoffmann-La Roche F,
4002 Basle, Switzerland

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Increased myocardial cathepsin D activity during regression of thyrotoxic cardiac hypertrophy

SEVERAL types of hormonal or mechanical stimuli may cause cardiac hypertrophy. In many instances, when the stimulus is removed the heart rapidly regresses toward its original size. The cellular mechanism(s) by which this net cardiac catabolism and reduction of myocardial protein mass might be mediated have not been defined. One possibility might be an increase in the activity of intracellular proteolytic enzymes; accordingly, we have investigated the activity of cathepsin D, a lysosomal enzyme that is the major acid proteinase in the heart.

Our experimental model was the hypertrophied heart of the thyrotoxic rat. Exogenous administration of excessive doses of thyroid hormone is well known to lead to marked cardiac enlargement in the rat, and discontinuation of thyroid treatment results in a rapid return of heart weight to normal¹⁻⁴. Indeed, during the first week of recovery from thyrotoxicosis, the heart loses 10% or more of its mass, in comparison with a weekly increase of 10% or more in normal rats⁴.

Two-month-old male albino rats were divided into four matched groups. The first group ('control') was untreated for 6 weeks. The second group ('thyrotoxic') received no treatment for 3 weeks followed by daily injections of *l*-thyroxine (1.0 mg kg⁻¹ intraperitoneally) for 3 weeks. The third group ('1-week recovery') received a similar 3-week course of thyroxine injections between the second and fifth week of the 6-week experimental period, and the last group was treated during the first 3 weeks only and then remained untreated for 3 weeks ('3-week recovery'). All the animals were killed on the same day by cardiac excision after being stunned by a blow to the head. Total body weights and the weights of the isolated cardiac ventricles were determined. A section of left ventricular muscle was excised immediately, diluted in a solution of 0.1% Triton at 4° C, minced and homogenised vigorously with a Willems homogeniser to disrupt maximally the cells and their organelles. The mixture was spun at 350g for

5 min to remove cellular debris, and the supernatant, which contained more than 90% of the total activity of the various enzymes tested, was taken for assay. Activities were measured for the lysosomal enzymes cathepsin D, β -acetylglucosaminidase and acid phosphatase^{5,6}. The activity of creatine phosphokinase⁷, a nonlysosomal enzyme that serves as a good general marker of myocellular viability, was also measured, as was the protein concentration⁸.

As others have described, thyroxine therapy caused marked cardiac hypertrophy in the rats. The ratio of ventricular weight to body weight was $0.36 \pm 0.01\%$ (s.e.m.) in the control group compared with $0.56 \pm 0.02\%$ in the thyrotoxic rats. The difference was due to a significant ($P < 0.01$) increase in ventricular weight as well as a decrease in body weight in the treated animals. One week after cessation of therapy, heart weight was significantly reduced and body weight was increased (ratio = $0.43 \pm 0.02\%$), and by 3 weeks all values had returned to normal (ratio = $0.38 \pm 0.02\%$).

These changes in thyroid state and heart size were accompanied by significant alterations in the cardiac activity of cathepsin D. With thyrotoxic hypertrophy there occurred a 15% decrease in the mean activity of the enzyme, from 68.7 to 58.4 μg tyrosine h⁻¹ mg⁻¹ protein ($P < 0.01$). Subsequently, with discontinuation of thyroid treatment and rapid regression of cardiac hypertrophy, the activity of the enzyme increased by 40% (Table 1) within 1 week. By 3 weeks, when the hearts had already returned to normal size, the activity of cathepsin D was also back to normal (71.3 μg tyrosine h⁻¹ mg⁻¹ protein).

During the rapid regression of hypertrophy, the increase in catheptic activity greatly exceeded the simultaneous decreases in heart weight and protein content (Table 1). Thus, the measured increase could not have been simply a consequence of slower net breakdown of the cathepsin D molecule compared with other cardiac proteins, which during generalised cardiac catabolism might lead to a relative elevation of the enzyme without an actual increase in its net production. Rather, the data indicate that there was an absolute increase in total cathepsin D activity in each heart, not due to generalised stimulation of all lysosomal enzymes. As Table 1 shows, there were no changes in the activities of glucosaminidase or acid phosphatase. Similarly, the nonlysosomal enzyme creatine phosphokinase was unaffected.

In separate tests, the tissue was homogenised gently in 0.25 M KCl to preserve intact lysosomes as far as possible^{9,10};

Table 1 Comparison of ventricular weight, protein, and enzyme activities.

	Thyrotoxic	1-week recovery
Body weight (g)	299 ± 7.6 (s.e.m.)	$339 \pm 6.5^*$
Ventricular weight (g)	1.66 ± 0.040	$1.43 \pm 0.024^*$
Ventricular protein concentration (mg g ⁻¹)	102 ± 1.4	$102 \pm 2.0^\dagger$
Creatine phosphokinase activity (μmol ATP per min per mg protein)	4.71 ± 0.145	$4.92 \pm 0.128^\dagger$
β -acetylglucosaminidase activity (nmol nitrophenol per h per mg protein)	140 ± 3.0	$138 \pm 3.1^\dagger$
Acid phosphatase activity (nmol nitrophenol per h per mg protein)	167 ± 5.3	$178 \pm 5.5^\dagger$
Cathepsin D activity (μg tyrosine per h per mg protein)	58.4 ± 1.05	$81.5 \pm 1.77^*$
Nonsedimentable (free) cathepsin D activity (% of combined free + particulate-bound activity)	30 ± 0.9	$35 \pm 1.4^*$

Measurements were made on hypertrophied hearts of 16 thyrotoxic rats and 16 hearts that were undergoing rapid regression of hypertrophy 1 week after discontinuation of thyroxine injections.

* $P < 0.01$ compared with thyrotoxic hearts.

$^\dagger P > 0.10$ compared with thyrotoxic hearts.

subsequent assays revealed altered catheptic activity in the 'free' or nonsedimentable fraction of the cell (which is thought by some to reflect enzyme that is more accessible to intracellular substrates *in vivo*) as well as in the total homogenate (Table 1).

These findings are reminiscent of other instances in which an organ responds to withdrawal of the appropriate hormone by an increase in the activity of lysosomal enzymes and catabolism and atrophy of the tissue^{11,12}. Of special interest is the regression of uterine smooth muscle after termination of pregnancy. Removal of the stimulus for uterine growth that is provided by sex hormones leads to rapid regression of the organ, and this is associated with an increase in the activity of uterine cathepsin D¹². In this condition there are parallel increases in other lysosomal enzymes, although in some other tissues pharmacological or hormonal changes cause specific alterations in cathepsin D without generalised effects on all lysosomal enzymes^{12,13}.

Our experiments demonstrate that acute changes in the thyroid state are accompanied by specific alterations in the activity of cathepsin D in the heart. Although no cause-and-effect relationship can be established definitively from our data, it seems reasonable to speculate that the observed changes in proteolytic activity are involved in the mediation of the corresponding shifts in protein balance. These findings provide for the first time evidence of an enzymatic alteration that might link an external stimulus for the regression of preexistent hypertrophy to subsequent myocellular catabolism. Whether similar changes accompany regression from other varieties of cardiac hypertrophy remains to be determined.

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KERN WILDENTHAL
EDWARD A. MUELLER

Pauline and Adolph Weinberger Laboratory for
Cardiopulmonary Research,
Departments of Physiology and Internal Medicine,
University of Texas Southwestern Medical School at Dallas,
Dallas, Texas 75235

Received February 8; revised March 28, 1974.

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Vasopressin release from the isolated neurohypophysis induced by a calcium ionophore, X-537A

TEN years ago observations on isolated neurohypophyses prompted the suggestion that action potentials in the hypothalamo-neurohypophysial tract release neurohypophysial hormones by promoting calcium influx and that the appearance of free calcium ions somewhere in the neurosecretory terminals provides the immediate stimulus for hormone extrusion¹⁻³. Some time later it was argued that the role of calcium ions in stimulus-secretion coupling in this system and in many other secretory cells was to set in motion a common secretory mechanism, exocytosis⁴. Very recently the evidence supporting this view has been strengthened by light microscopic observations showing extrusion of secretory granules from mast cells on intracellular injection of calcium ions⁵. Meanwhile, electron microscopic⁶⁻¹¹ and chemical¹²⁻¹⁶ evidence has borne out the conjecture that neurohypophysial hormones are secreted by exocytosis, and the case for calcium-influx as the initiating event has been strengthened by a number of observations set out in recent reviews^{15,16}. These reviews make the further point that the neurohypophysis may be a useful model system for studying stimulus-secretion coupling in nerve cells generally: the involvement of calcium in release of neural transmitters is well-known and may have a similar explanation^{17,18}.

With the discovery of calcium ionophores a new tool has become available, and our purpose here is to report the stimu-

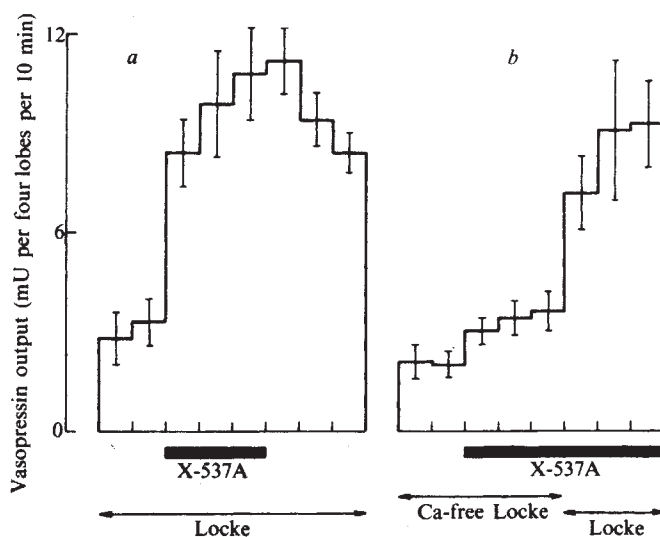


Fig. 1 Stimulant effect of the ionophore X-537A on output of vasopressin from rat neurohypophyses *in vitro* and the inhibitory effect of calcium deprivation. The methods for isolating neurohypophyses, incubating them, and measuring vasopressin output followed the original description² except that four neural lobes from male rats weighing about 250 g were used in each experiment and were impaled, after halving, on a fine platinum wire holder²⁹ facilitating transfer from one incubation chamber to the next. Media containing X-537A were prepared from a stock solution of the drug in dimethylsulphoxide. The final concentration of the solvent never exceeded 0.2% which is without effect on basal or stimulus-evoked output of vasopressin³⁰. Drug and solvent did not interfere with the rat blood pressure method used for bioassay of vasopressin. Each of the two curves (a) and (b) shows vasopressin outputs (means $\pm$ s.e.) in successive 10 min incubation periods in three experiments. X-537A ($6 \mu\text{g ml}^{-1}$) was present during the periods indicated by the heavy horizontal bars. Horizontal arrows indicate the incubation medium. Locke's solution contained (mM): NaCl, 150; KCl, 6.0; CaCl_2 , 2.0; MgCl_2 , 1.0; NaHCO_3 , 10; glucose, 10. It was equilibrated with 5% CO_2 in O_2 . In calcium-free Locke, CaCl_2 was omitted. Records begin after 40 min preincubation with Locke's solution (a) or calcium-free Locke's solution (b).

Table 1 Effect of calcium deprivation, with or without EDTA, on output of vasopressin in response to X-537A (25 $\mu\text{g ml}^{-1}$)

Preincubation	(A) Locke (30 min)	(B) Ca-free Locke (60 min)	(C) Ca-free Locke + 2 mM EDTA (60 min)	(D) Ca-free Locke + 2 mM EDTA (70 min) followed by Mg-free Locke (60 min)
Incubation medium	Locke	Ca-free Locke	Ca-free Locke	Mg-free Locke
Vasopressin output (mU per four lobes per 10 min: mean $\pm$ s.e.m. (n))				
Before X-537A	2.0 $\pm$ 0.3(5)	2.1 $\pm$ 0.3(4)	2.7 $\pm$ 0.2(4)	2.2 $\pm$ 0.4(4)
During X-537A*				
0–10 min	9.1 $\pm$ 1.5(5)	4.8 $\pm$ 0.8(4)	3.6 $\pm$ 0.4(4)	4.7 $\pm$ 0.5(4)
10–20 min	13.5 $\pm$ 1.7(5)	4.9 $\pm$ 0.6(4)	3.8 $\pm$ 0.4(4)	6.8 $\pm$ 0.6(4)
20–30 min	16.7 $\pm$ 2.3(4)	4.9 $\pm$ 0.6(4)	3.8 $\pm$ 0.1(3)	8.5 $\pm$ 1.2(4)

* All outputs (columns A–D at each 10 min interval) are significantly higher ($P < 0.05$) than the corresponding values before X-537A.

lant effects, on neurohypophysial secretion, of one of these, X-537A. Like other calcium ionophores, X-537A is a substance that forms a reversible complex with calcium which, by its lipophilic nature, serves as a mobile calcium carrier that will transport calcium ions across biological membranes¹⁹. It is known to release histamine²⁰ and, more relevant to the work reported here, to elicit extrusion of secretory granules from mast cells by calcium-dependent exocytosis^{21,22}.

The methods we have used for studying release of vasopressin from isolated rat neurohypophyses have been described elsewhere (see legend to Fig. 1). In a concentration of 6 $\mu\text{g ml}^{-1}$, X-537A increased vasopressin output about three-fold in preparations incubated in a conventional, calcium-containing, Locke's solution. The effect outlasted exposure to the drug (Fig. 1a). By contrast, this ionophore had little stimulant effect on preparations incubated in calcium-free Locke, although vasopressin output rose substantially when calcium was reintroduced (Fig. 1b). This stimulant effect of calcium was clearly attributable to some priming effect of X-537A because calcium had no detectable effect on vasopressin output in each of four experiments where it was reintroduced after the same period of calcium deprivation without exposure to the ionophore, a finding in agreement with earlier observations².

In a concentration of 25 $\mu\text{g ml}^{-1}$, X-537A evoked a still greater output of vasopressin; the response in the presence of calcium (Table 1A) being approximately equal to that yielded by a maximally effective concentration (60 mM) of potassium². Omission of calcium (with or without addition of EDTA) inhibited the response (Table 1B and C). This inhibitory effect was reversed, at least in part, by reintroducing calcium without magnesium (Table 1D).

From these results it is evident that X-537A can stimulate vasopressin output through some calcium-dependent process. The most obvious explanation is that the drug acts on the neurohypophysial terminals as a calcium ionophore, and allows calcium to penetrate these terminals and initiate secretion in line with the original calcium influx hypothesis¹. But at least one alternative, or additional, explanation deserves consideration, namely that X-537A owes its effect on vasopressin release to its additional ability to complex with and transport monovalent cations¹⁹. By such an action the drug might depolarise the neurosecretory terminals and set in motion the familiar potential-dependent calcium influx mechanism^{3,23}.

Further experiment revealed an additional stimulant effect of X-537A on vasopressin output which is not readily explained in terms of depolarisation or calcium entry. Thus, in a concentration of 50 $\mu\text{g ml}^{-1}$ the drug increased vasopressin output from neurohypophyses exposed to a depolarising² concentration (60 mM) of potassium in a calcium-free medium (Fig. 2). The response was about half that subsequently obtained in the same preparations 10–20 min after introducing 2 mM calcium in the presence of 60 mM potassium which, as noted above, is the most effective concentration of potassium for evoking vasopressin secretion². In the light of evidence that X-537A can release calcium from sarcoplasmic reticulum^{24–28}, we suggest that this stimulant effect on output

of neurohypophysial hormone may result from mobilisation of cellular calcium and consequent elevation of the concentration of free calcium ions in the neurosecretory terminals. This would be in line with the original suggestion that the appearance of an excess of free calcium ions somewhere in the terminals provides the signal for release of neurohypophysial hormones¹.

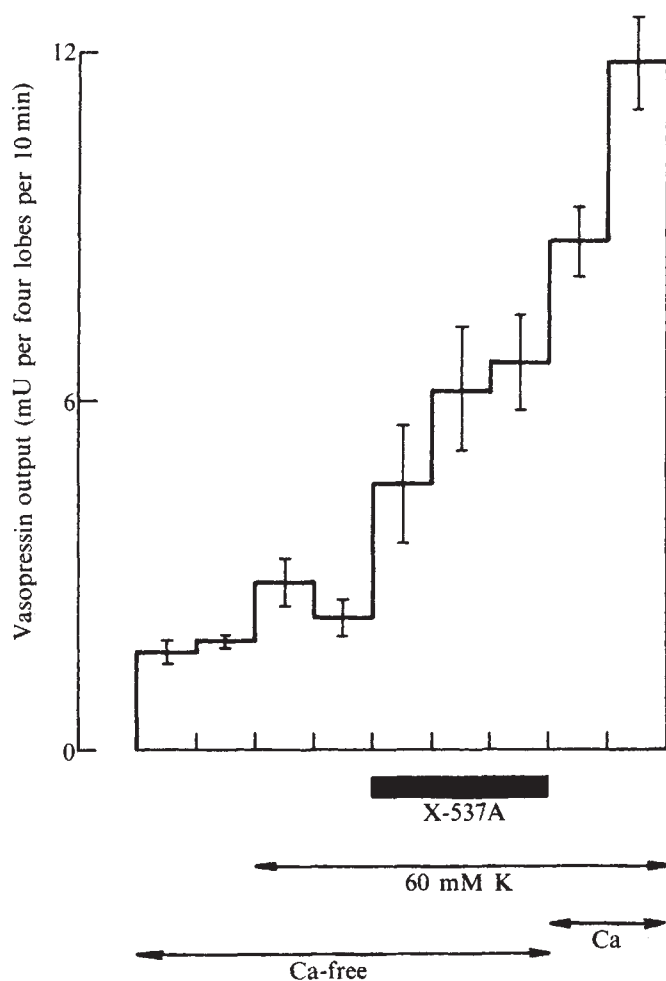


Fig. 2 Stimulant effect of the ionophore X-537A (50 $\mu\text{g ml}^{-1}$) on vasopressin output from neurohypophyses incubated in calcium-free Locke containing a depolarising² concentration of potassium. The record represents the mean ($\pm$ s.e.m.) of four experiments and begins with lobes in calcium-free Locke (Ca-free) after 40 min preincubation in this same medium. After the first two periods shown, incubation was continued with calcium-free Locke with the potassium concentration raised to 60 mM ('60 mM K'), the sodium concentration being lowered correspondingly to maintain tonicity². X-537A was present during the period indicated by the heavy horizontal bar. After exposure to the drug, incubation was continued with 60 mM K Locke with the addition of 2 mM calcium (Ca). The secretory response obtained on adding calcium is near maximal for such preparations².

Conjecture along these lines is interesting because little is known of the physiological mechanisms for binding or sequestering calcium in secretory systems; yet some such mechanism must exist, as in muscle, if calcium is to function as a mediator in stimulus-secretion coupling. It is conceivable that responses to ionophores obtained from secretory preparations in calcium-free environments may provide a clue. It is possible however that X-537A, especially in the higher concentrations, may be able to release vasopressin independently of cellular as well as extracellular calcium: there are indications that acetylcholine can be released from nerves without involvement of calcium—for example, by high concentrations of ethanol³¹.

In contrast to X-537A we have found, in similar experiments on six batches of neurohypophyses, that the calcium ionophore A-23187, in a concentration of 1, 2, 5 or 50 $\mu\text{g ml}^{-1}$, caused no obvious increase in vasopressin output. Since A-23187 differs from X-537A in having relatively little affinity for monovalent cations¹⁹ this could be interpreted as support for the view that the response to X-537A involves depolarisation as well as calcium. Alternatively, A-23187 may for some reason, fail to act as an effective calcium ionophore in the neurohypophysial preparation as we have studied it. In any event, the behaviour of the neurohypophysis is quite different from that of mast cells studied in this laboratory where A-23187 (2–3 $\mu\text{g ml}^{-1}$) as well as X-537A causes a prompt release of secretory granules by exocytosis^{21,22}. It is thus evident that different secretory cells vary in responsiveness to different ionophores as they do to various other stimuli¹⁶. Discussion of the possible physiological significance of this difference is best deferred until evidence from various other secretory preparations has been presented. These initial results encourage us to believe, however, that calcium ionophores may be useful new tools for the study of stimulus-secretion coupling in nervous tissue. We would add only, with regard to the exocytosis hypothesis of release of neurohypophysial hormones, that a stimulant effect of calcium after priming with X-537A, such as we have described for the neurohypophysis (Fig. 1b), has also been observed in mast cells where light and electron microscopy has shown the response to be extrusion of secretory granules by exocytosis^{21,22}.

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Y. NAKAZATO
W. W. DOUGLAS

Department of Pharmacology,
Yale University School of Medicine,
333 Cedar Street,
New Haven, Connecticut 06510

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Capillary permeability and sympathetic activity in canine subcutaneous adipose tissue

PREVIOUS studies on the exchange properties of the microvasculature of canine subcutaneous adipose tissue have shown that electrical stimulation of the sympathetic nerves with frequencies within the physiological range, induced vasoconstriction accompanied by an increase in the trans-capillary hydrodynamic conductivity expressed by the capillary filtration coefficient (CFC) (ref. 1). Given that CFC is a function of the product of the surface area available for exchange and the permeability of the capillary wall^{2,3}, these two factors must be separated to establish the functional properties of this vascular reaction. In attempting to do this, the clearance of locally injected Na¹²⁵I was measured at constant flow perfusion, and was found to be significantly reduced during sympathetic stimulation. Similarly the permeability-surface area product (PS) of the vascular bed measured by the extraction of ⁸⁶Rb in subcutaneous adipose tissue decreased as a function of sympathetic stimulation (ref. 4 and J. Gainer, B. Linde, and S.R., unpublished).

These data suggest that sympathetic stimulation causes a constriction of the precapillary vessels resulting in a diminished number of capillaries open to blood flow. The increase of CFC could then be a consequence of either an overall increase in permeability of the capillary membrane, or a drastic alteration of the capillary pressure profile, which would enhance the effect of the more permeable venous segments of the microvasculature⁵.

The present study was undertaken with the idea that a higher capillary permeability might affect the isovolumetric capillary pressure (P_{ci}) provided that the permeability change is of such a nature that osmotically active molecules become diffusible across the capillary membrane that is, the osmotic reflection coefficient for plasma proteins becomes smaller than one⁶.

To test this hypothesis, experiments were performed on 13 female, mongrel dogs, anaesthetised with sodium pentothal

(30 mg kg⁻¹). The inguinal subcutaneous adipose tissue, located between the pubis and mammary glands, was prepared on the right side and placed in a plethysmograph with the artery, vein and nerve passing through a closely fitted opening^{1,7}. After the administration of heparin, the artery supplying the adipose tissue was cannulated with plastic tubing and a silicone filled drop recorder was inserted. The plethysmograph was filled with Tyrode's solution kept at 38° C and connected to a volume recorder. Volume changes could be determined with an accuracy of ± 0.05 ml. Blood pressures were monitored from the artery and vein supplying the adipose tissue by means of Statham pressure transducers and recorded on a Grass polygraph, together with the blood flow and tissue volume. The vein draining the tissue was cannulated with a widebore plastic tubing, the free end of which could be placed at any desired level above the preparation. In this way the venous outflow pressure, and therefore the venous and capillary pressures in the adipose tissue, could be varied. The isovolumetric capillary pressure (P_{ci}) was determined according to the principle of Pappenheimer and Soto-Rivera⁸. The blood flow was reduced in a stepwise fashion by means of a screw clamp on the artery. The level of the venous cannula was then adjusted to give a hydrostatic capillary pressure which rendered the adipose tissue isovolumetric. The values of arterial and venous pressures at isovolumetric states were then plotted (Fig. 1) and P_{ci} was estimated by extrapolating to zero pressure difference.

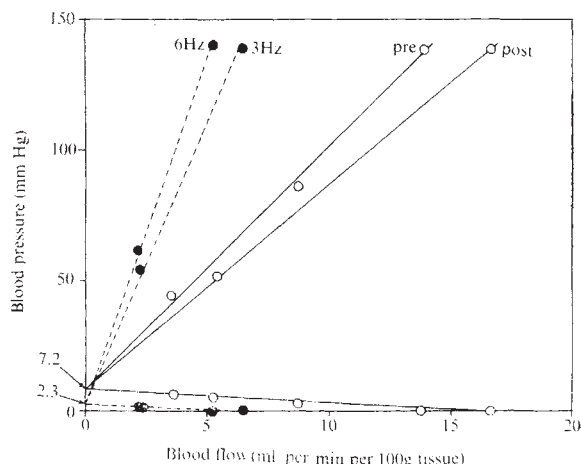


Fig. 1 Determination of isovolumetric pressure in canine subcutaneous adipose tissue. The points indicate arterial or venous pressures at different levels of blood flow at isovolumetric states. O, Determinations made during resting conditions (control), pre- and post-stimulation respectively; ●, determinations made during electrical stimulation of sympathetic nerves with 3 and 6 Hz, respectively. The data are extrapolated linearly to zero pressure difference, which coincides with zero blood flow. P_{ci} is indicated by the intercept on the ordinate. Colloid osmotic pressure 180 mm Hg.

In all experiments the extrapolated arterio-venous zero pressure difference was found to coincide with zero blood flow, as shown in Fig. 1. At zero flow there is no pressure drop along the blood vessels and, consequently, P_{ci} is equal to the pressure at the intercept of the extrapolated pressure-flow relations on the ordinate. The linear relationship between isovolumetric blood flow and isovolumetric arterial and venous pressures, respectively, is evidently a consequence of the lack of autoregulatory responses in subcutaneous adipose tissue. Therefore, this method can be used with some precision to determine P_{ci} in subcutaneous adipose tissue.

The mean resting isovolumetric capillary pressure was 10.1 mm Hg (range 20.8–3.8, $n=13$), which was found to

be lower than the plasma colloid osmotic pressure, 17.6 mm Hg (range 18.3–15.6). (The plasma colloid osmotic pressure was determined by means of a Hansen type osmometer utilised in conjunction with a membrane whose reflection coefficient for albumin was one.) Thus, the hydrostatic pressure which counterbalances all pressures tending to cause fluid absorption at an isovolumetric state is consistently lower than the plasma colloid osmotic pressure by an average of 7.6 mm Hg. This finding may be accounted for by a tissue osmotic pressure of 5–8 mm Hg which corresponds to a tissue protein concentration of 2–3%. This was shown to exist by direct micro-sampling in subcutaneous connective tissue⁹, and by measuring the distribution of tagged albumin¹⁰.

Following sympathetic nerve stimulation (3–9 Hz; 2 ms; 7–10 V), which was adjusted to diminish the blood flow to approximately one half, there was a marked reduction of P_{ci} , which averaged 5.6 mm Hg (range 10.8–0; $P<0.001$) (Fig. 1). As in the case of resting conditions, in every experiment it was possible to determine P_{ci} by linear extrapolation to zero flow. P_{ci} is estimated by gradually decreasing the arterio-venous pressure difference. In this manner, uneven flow distributions in the vascular bed are diminished. Therefore reduced P_{ci} does not seem to be the effect of a redistribution of blood to areas of higher permeability.

In view of the above mentioned changes in CFC and PS, the reduction of P_{ci} is compatible with an increase in capillary permeability associated with a decrease in the reflection coefficient for plasma proteins. These experiments thus support the hypothesis that the sympathetic nervous system may control capillary permeability in canine subcutaneous adipose tissue¹¹. Adrenergic vasomotor control of permeability has not been described previously.

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MARCOS INTAGLIETTA

AMES-Bioengineering,
University of California, San Diego,
La Jolla, California 92037

SUNE ROSELL

Karolinska Institutet,
Department of Pharmacology,
104 01 Stockholm 60, Sweden

Received December 31, 1973.

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Intracellular electric responses to sound in a vertebrate cochlea

ELECTRIC potentials generated by the inner ear in response to sound stimulation were first recorded 40 yr ago¹. Studies

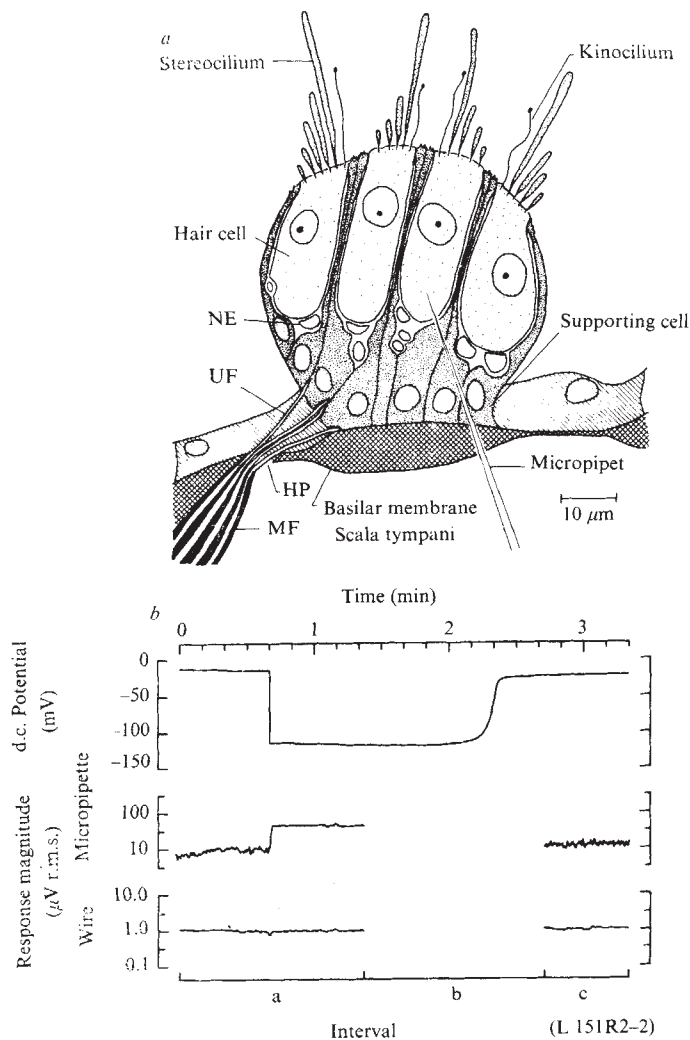


Fig. 1 *a*, Drawing of a transverse section through the basilar papilla of the alligator lizard. The basilar papilla is approximately 50 μm wide by 400 μm long and contains about 150 hair cells innervated by approximately 600 nerve fibres²⁴⁻²⁶. The penetration of the basilar papilla from scala tympani by a micropipette is shown schematically for a typical experiment. HP, habenula perforata; MF, myelinated portion of a nerve fibre; NE, nerve ending; UF, unmyelinated fibre. *b*, Oscillographic records obtained as a micropipette was advanced into the papilla. The top and centre traces were obtained from the potential recorded between the micropipette and a remote reference electrode. The top trace shows the d.c. potential; the centre trace shows the magnitude of the response to a 500 Hz tone at a sound pressure level (SPL) at the tympanic membrane of 75 dB. The bottom trace is the magnitude of the response to the 500 Hz tone obtained from a wire electrode near the round window. For both the centre and bottom traces the potential recorded from the electrode was passed through a narrow-band filter (2 Hz bandwidth) to obtain the fundamental component of the response, the magnitude of which is plotted on a logarithmic ordinate scale²⁷. During intervals *a* and *c*, the 500 Hz tone was on continuously; during interval *b*, this tone was turned off and responses to other acoustic stimuli were recorded. When the d.c. potential suddenly dropped to -110 mV, the micropipette tip was assumed to have entered a cell, and the micropipette was held stationary. Coincident with the occurrence of the negative d.c. potential, the response magnitude increased abruptly by at least a factor of 4. After the d.c. potential returned to near zero, the magnitude of the response decreased almost to its previous value. The bottom trace shows that throughout the penetration by the micropipette into the basilar papilla, the response recorded from the wire electrode remained constant.

of these extracellular potentials have led to hypotheses about the role of intracellular potentials in sensory transduction in receptor (hair) cells^{2,3}. It is difficult, however, to infer these intracellular potentials from extracellular measurements alone⁴⁻⁷; measurements of intracellular potentials from hair cells are clearly desirable. Although intracellular potentials in other hair-cell receptors have been reported recently^{8,9}, the few previous attempts to record such potentials in the cochlea have not yielded significant results¹⁰. We report here intracellular responses from single hair cells and supporting cells in the cochlea of the alligator lizard *Gerrhonotus multicarinatus*.

The lizard ear is basically similar to the mammalian ear^{11,12}. Sound causes vibrations of the tympanic membrane which are transmitted by a cartilaginous extra-stapes and a bony stapes to the fluids of the inner ear, where the basilar membrane is set in motion. The auditory receptor organ (basilar papilla) consists of a strip of hair cells and supporting cells, which is on the basilar membrane (Fig. 1*a*). The basilar papilla is generally considered to be homologous^{13,14} to the mammalian organ of Corti.

In our experiments, some bone and the round window membrane were removed so that the papilla could be visualised with a dissecting microscope. As a glass micropipette (tip diameter less than 0.5 μm) was advanced into the papilla (Fig. 1*a*) sound was delivered to the external ear and electric potentials were measured continuously. An abrupt negative shift in the d.c. potential was taken to indicate that the tip of the micropipette had entered a cell (Fig. 1*b*). (Intracellular d.c. potentials ranged between -25 mV and -125 mV in 90% of 547 cases with a median of -74 mV.) Stable negative d.c. potentials were often recorded for as long as 2 min and in a few cases for 30 min. The maximum displacement of the basilar membrane in these experiments (measured by the Mössbauer method¹⁵) was estimated to be less than 0.1 μm . Since this displacement is much smaller than the dimensions of the cells, it is not surprising that the micropipette tip could remain intracellular in the presence of this motion. Attempts to mark the cells after recording their responses to sound were made by passing current through dye-filled micropipettes^{16,17}. Immediately after the experiment the intact papilla was dissected and the position and type of each dye-marked cell were determined by observation with a light or fluorescence microscope. For more precise localisation of the dye, the basilar papilla was sometimes embedded in Epon and serial sections were examined.

To assess whether the experimental procedures interfered grossly with the normal operation of the basilar papilla, extracellular responses were measured with a wire electrode on the bone near the round window. It was possible to expose the basilar membrane and to advance a micropipette into the papilla without significantly changing this extracellular response (Fig. 1*b*, bottom trace). The electric responses recorded intracellularly apparently did not result from mechanical vibration of the micropipette tip, because injection of sodium pentobarbital into scala tympani greatly reduced both the intracellular responses recorded with the micropipette and the extracellular response recorded with the wire electrode without changing the motion of the basilar membrane.

The intracellular responses to sound recorded in the papilla were graded potentials with a maximum amplitude of 3 mV peak-to-peak, and a short (less than 0.5 ms) latency in response to clicks. Responses to tone bursts had component waveforms that resembled the cochlear microphonic (CM) and summing (SP) potential that have been recorded extracellularly^{2,18}, suggesting that these intracellular response components (ICM and ISP in Fig. 2*a*) may be sources of the extracellularly recorded responses. Although the ratio of the magnitudes of ISP and ICM varies from cell to cell and is a function of stimulus parameters, ISP

was always a depolarisation. ICM was approximately sinusoidal with a fundamental frequency equal to the tone frequency (Fig. 2b).

Electric responses to sounds were of nearly the same magnitude in both hair cells and supporting cells, but the waveforms of the responses to clicks differ in these two types of cells¹⁹. The largest responses recorded (3 mV) from identified hair cells in the basilar papilla were comparable in magnitude with those reported for identified hair cells in lateral-line organs^{8,9}. In contrast, the responses recorded in supporting cells were apparently larger (1.5 mV maximum) than those found in lateral-line organs^{8,9}. All-or-none

(action) potentials were never observed during penetrations of the basilar papilla, but were recorded during penetrations of the myelinated portion of the cochlear nerve at locations between the ganglion cell bodies and the habenula perforata (Fig. 1a).

We should like to make some general comments about our results: (1) The existence of intracellular response potentials which are similar in waveshape but larger than the extracellular cochlear microphonic and summing potentials is consistent with the widely-held view that the cells of the sensory organ are sources of these extracellular potentials. The sodium pentobarbital-induced reduction of both intracellular and extracellular potentials is also consistent with this view. (2) The occurrence of responses of comparable magnitude in hair cells and supporting cells suggests either that supporting cells respond directly to sound and/or that hair cells are sources of currents which produce potentials in supporting cells. Thus supporting cells may play some role in the transduction process¹⁹. (3) The hair-cell afferent nerve junction is probably a chemical synapse³. Studies of chemical synapses indicate that pre-synaptic potentials of a few millivolts can produce changes in the release of transmitter substances^{20,21}. Thus the receptor potential in hair cells may modulate transmitter release. (4) Studies of action potentials in single fibres of the auditory nerve^{22,23} have demonstrated that during a tone burst (a) the mean rate of occurrence of action potentials is increased, and (b) action potentials tend to occur in synchrony with individual cycles of the tone. The fact that intracellular potentials in receptor cells have components (ISP and ICM) with waveshapes that are similar to the observed action-potential patterns is consistent with the view that these intracellular potentials may be a causal step in the excitation of action potentials in auditory nerve fibres.

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M. J. MULROY*
D. W. ALTMANN
T. F. WEISS†
W. T. PEAKE†

Eaton-Peabody Laboratory of Auditory Physiology,
Massachusetts Eye and Ear Infirmary,
Boston, Massachusetts 02114

* Also at Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115.

† Also at Research Laboratory of Electronics and Department of Electrical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

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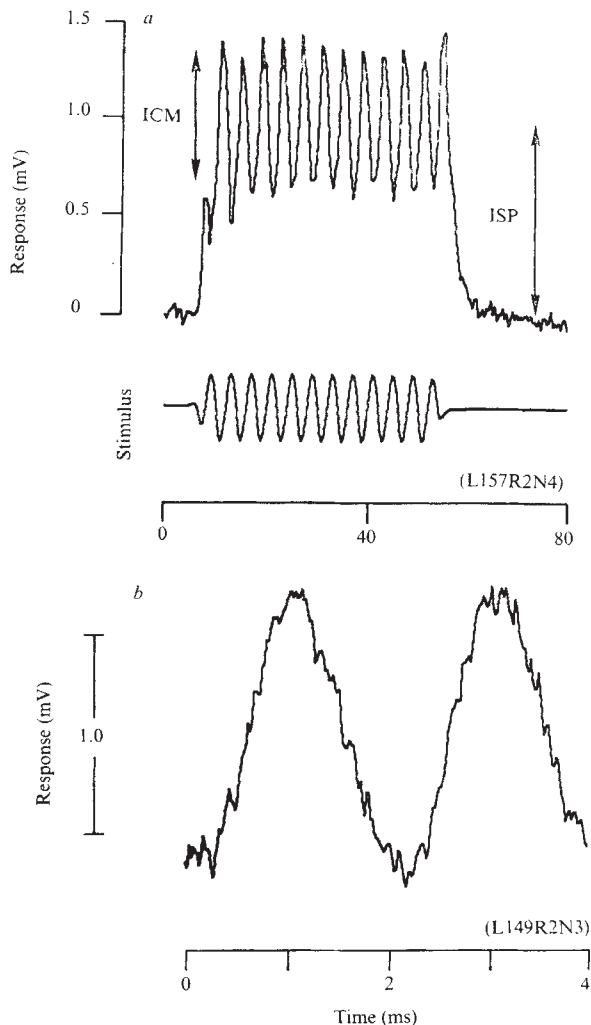


Fig. 2a, Averaged intracellular response to tone-burst stimuli. Deviations of the potential from the resting potential are shown; upward direction is a depolarisation of the cell. The cell in which this response was recorded was identified as a supporting cell based on its response to acoustic clicks¹⁹. ICM (intracellular cochlear microphonic) is the response component that follows the individual cycles of the tone: ISP (intracellular summing potential) follows the tone burst envelope. The arrows indicate the approximate magnitudes of these components. Tone bursts of 50 ms duration were delivered at a rate of 10 s⁻¹. Rise-fall time was 2 ms. Frequency was 250 Hz and level was 94 dB SPL at the tympanic membrane. The average response was computed from 333 individual responses sampled at 400 μ s intervals at 200 points. These points are connected by straight lines. **b**, Averaged intracellular response to a continuous tone recorded from a hair cell (as identified by dye-marking). Tone frequency was 500 Hz and level was 73 dB SPL. This averaged response was computed from 310 individual responses sampled at 20 μ s intervals.

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Behavioural and physiological bases of drinking inhibition in water deprived rats

FLUID balance is normally precisely maintained. Yet, following water deprivation, neither rat nor man drink enough water to correct the incurred fluid deficit. Fluid balance is corrected, however, when food is also made available. Adolph¹ termed this phenomenon voluntary dehydration.

In this communication, experiments that identify the physiological and behavioural bases of voluntary dehydration are described. In preliminary analyses, to be reported elsewhere, we found first, that only 79% of the fluid lost by rats deprived of water for 24 h was restored by drinking; second, that water intake stopped in spite of a persistent intravascular deficit (estimated to be 20% from haematocrit and plasma protein measures²); and third that the cessation of drinking was closely associated with the development of cellular overhydration (based upon Leaf's treatment³ of Na as an estimator of plasma volume). Specifically, 15 min after the start of drinking, 79% of drinking was complete and the cellular phase was estimated to be 1.5% overhydrated ($t = 2.217$, $d. f. = 15$, $P < 0.05$).

These correlational observations indicate the following alternatives regarding the determinants of voluntary dehydration. Intake may be exclusively controlled by the hydrational status of the cellular phase. That is, cellular overhydration may be the necessary and sufficient event to prematurely terminate drinking. This predicts that the manner in which cellular overhydration is attained by drinking compared with gavage, is irrelevant. Absorption may allow the mouth and stomach to control drinking either by metering fluid intake or by reducing the urge to drink. Both oral and postgestional information may be critical and drinking may stop because the ingestive act is closely related in time to its postgestive consequences.

To distinguish among these alternatives, ten adult female Sprague Dawley rats were each studied on nine occasions (including two baseline tests where sham preloads were given).

Table 1 Outline of deprivation, preloading and drinking schedule followed in this study

0 h	23 h	24 h	25 h
Water deprived	Preload	Preload	Preload
(Purina pellets available)	Intubate or drink water or 0.153 M NaCl*	Intubate or drink water or 0.153 M NaCl*	Drink water or 0.153 M NaCl for 5 h

* The amount given was 27.5% of the water loss.

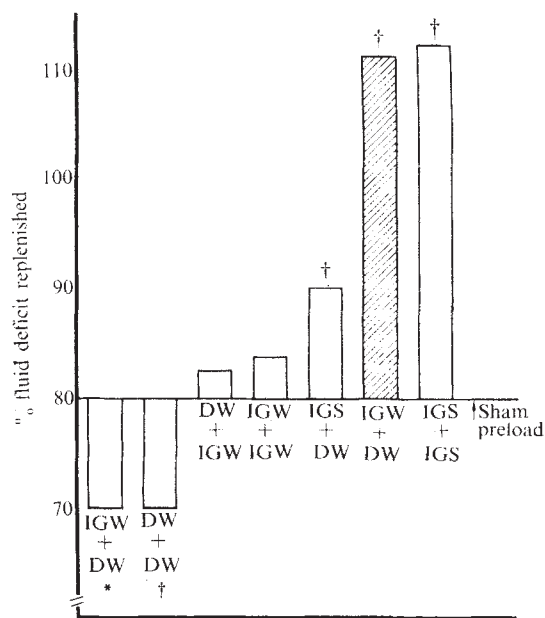


Fig. 1 Percentage of fluid deficits replenished in each of the eight conditions. % replenished = [(preload (ml) + test intake (ml))/water loss (ml)] $\times$ 100. The spontaneous drinking of sham loaded rats is presented as the baseline at 80%. IGW, Intragastric water; DW, drinking water; IGS, intragastric saline; DS, drinking saline. $\square$ Water drunk during test; saline drunk during test. $\square$ The first open column, for example, indicates that the first preload was water delivered intragastrically, water was drunk as the second preload and water was drunk during the 5 h test. * $P < 0.05$ † $P < 0.01$

Tests were separated by a mean of 7 d (range 4–10 d) and each rat was tested under a different semirandom order of preload and drinking conditions. The general procedure is presented in Table 1. On a given test, following 23 h of water (but not food) deprivation, food was removed and each rat drank, or received by gavage, one half of its fluid loss (approximated to be 67% of body weight loss⁴). One hour later the second half was either drunk or intubated. After another hour, either tap water or isotonic saline (0.153 M) was made available for 5 h. Food and water were then made continuously available until the next test.

Figure 1 compares total intakes (that is, the amount of preload and amount drunk during the drinking test) to the amount drunk spontaneously following sham loadings. In all but two conditions, total intake equalled or exceeded the amount drunk spontaneously following sham loadings. Total intake was actually reduced in the two exceptional cases (Fig. 1) and it was only in these two conditions that the second water preload was drunk following an initial water preload. Absorption of this volume of water was sufficient to produce cellular overhydration. Thus, only in situations in which development of cellular overhydration was closely paired with drinking, was premature termination of drinking exaggerated.

Cellular overhydration *per se* was not the critical event terminating drinking. If it were then the outcomes of the first four conditions, in which identical water preloads were delivered, and comparable haemodilution occurred, should not have differed one from another.

Neither the amount of the preload drunk nor the time spent in its drinking were the determining factors. If they were, then intake following the DW+DW sequence (see Fig. 1) should have been the most suppressed. Drinking here did not differ however, from the IGW+DW condition where only one half of the preload was drunk. Moreover, if oral metering *per se* were the critical factor, then intake following IGW+DW should not have differed at all from that following the DW+IGW sequence. As seen in Fig. 1, however, intakes differed markedly.

The IGS+DW condition (Fig. 1) indicates that the prevailing factor in the first two conditions was not the memory of having recently drunk water. Here the preload of DW followed intragastric isotonic saline. That is, drinking occurred in the absence of cellular overhydration. This emphasises that when water intake is divorced from cellular overhydration, drinking continues.

The shaded column of Fig. 1 (the test solution isotonic saline) suggests that the efficacy of treatment IGW+DW resided in the inhibition of water ingestion and not in thirst reduction. If it were the elimination of thirst, then no drinking should have occurred during the test hour regardless of the proffered solution. Yet a considerable volume of saline was ingested presumably in response to the prevailing extracellular deficit. This is an important aspect of the phenomenon for it raises the possibility that inhibition of drinking is specific to the solution drunk during the development of cellular overhydration. The final column emphasises the importance of cellular overhydration. When the entire preload was isotonic saline, a solution which has little initial effect on cellular balance, water intake was very substantial (9.4 ml).

The only conditions that exaggerated voluntary dehydration were those in which water intake occurred in contiguity with cellular overhydration; namely, drinking the entire water preload or intubating the first half of the preload and drinking the second half. Intubation of the entire preload or drinking the first half and tubing the second half did not have this effect.

These data provide the behavioural and physiological bases for the natural occurrence of voluntary dehydration in rats and indicate that: as drinking ensues, water is quickly cleared from the stomach and absorbed⁴; the cellular phase becomes rapidly overhydrated, and this occurs while the rats are actively engaged in drinking; the water remaining in the stomach and intestine is cleared, absorbed, and excreted in a dilute urine. The exaggeration of voluntary dehydration is likely a result of allowing ample time for complete absorption following the preloads and thereby avoiding any lag in the system.

The above theory predicts therefore, that even if the entire amount drunk were to be preloaded by gavage, drinking would still occur. This is precisely what happened. Ten rats preloaded a mean of 14.7 ml (the gross intake of a previous test) drank an additional 3.0 ml (range 1.0–5.0 ml) despite the fact that the preload caused substantial overhydration ($\bar{X}=7.1\%$).

The implications of this report may extend beyond voluntary dehydration to include other ingestive phenomena. For example, Nachman and Valentino⁵ have shown that adrenalectomised rats, although intubated with the amount of NaCl required to satisfy their salt deficit, drank a considerable amount of 0.3 M NaCl. There have also been reports that water delivered by gavage is not nearly as effective as water taken by mouth in reducing intake to either intravascular depletion by hyperoncotic colloid⁶ or to intracranial angiotensin⁷. According to Fitzsimons, rats infused with their normal daily water requirement still drink significant amounts⁸. Janowitz and Grossman⁹ have shown that loading food intragastrically 20 min before regular feeding failed to depress intake in dogs. Baille *et al.*¹⁰ reported that intragastric intubation of glucose inhibited feeding only when the load was delivered concurrently with feeding. If the load was delivered 30 min in advance of feeding, there was no suppression. Miller¹¹ has presented extensive evidence that rats work for more food or water following intragastric preloads than following oral preloads. These data have, in general, been interpreted from the 'drive stimulus reduction' point of view, or with the idea that the mouth in some way meters the amount of ingesta appropriate to the animals' requirement. In light of the present evidence, these interpretations might be re-evaluated and the influence of ingestion in contiguity with its postingestional consequences explored.

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ELLIOTT M. BLASS
WARREN G. HALL

Department of Psychology,
Johns Hopkins University,
Baltimore, Maryland

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Optical polarisation indicates linear arrangement of rhodopsin oligosaccharide chain in rod disk membranes of frog retina

RHODOPSIN, the visual pigment of the retinal rods, is a doubly conjugated protein constituting about 80% of the total protein of the rod disk membrane in the frog¹. The two prosthetic groups of rhodopsin are the visual chromophore retinal, and a single oligosaccharide chain comprising six sugar residues^{2,3}.

Electron microscope^{4,5,6} and X-ray diffraction^{7–10} studies offered no evidence for the orientation of the chromophore molecules or the oligosaccharide chains of rhodopsin with respect to the plane of the disk membrane. The negative, dichroism of the outer segments with the greater absorption for light polarised perpendicularly to the rod axis^{11,12} indicated that the chromophore molecules are oriented with their long axis parallel to the plane of the disk membranes. That no dichroism in the axial direction could be observed, indicates that the chromophore molecules are scattered randomly in all directions with their lengths in the plane of the membrane. It was found recently^{14,15} that when a flash of linearly polarised light passed along the lengths of the rods, axial dichroism was induced as a result of bleaching of the retinal molecules lying parallel to the plane of polarisation

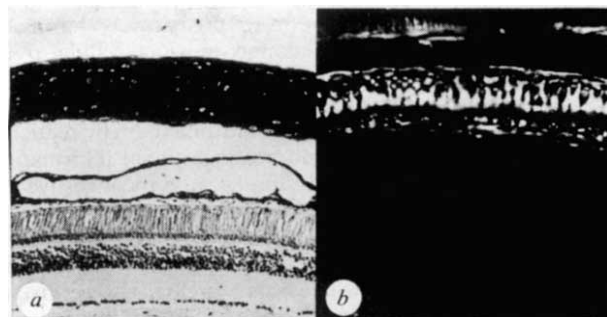


Fig. 1 Paraffin section of formaldehyde-fixed frog retina, depigmented with H_2O_2 and stained with 0.1% toluidine blue at pH 3.0 for 5 min. After blotting off the dye solution, the staining effect was stabilised with 2% potassium ferricyanide²⁰ and mounted in gum arabic which was allowed to dry; *a*, in the light microscope, at this pH, only the cell nuclei appear stained the rod outer segments show no dye binding. At the top the basophilic scleral cartilage is seen; *b*, the same field with the polaroids crossed. The ground substance of the cartilage shows strong toluidine blue induced birefringence²⁰. Above the cartilage birefringent collagen fibres are seen.

of the light used. This dichroism, however, was very short lived and rapidly decayed with a relaxation time of about 12 ms, but was more persistent in glutaraldehyde-fixed rods. It was concluded that in the native state rhodopsin molecules were free to undergo rotational (Brownian) motion, however, with the chromophore remaining in the plane of the disk membrane. This would indicate a relatively fixed rotation axis of the rhodopsin molecules perpendicular to the plane of the membrane.

Thus rhodopsin seems to be highly oriented in the photoreceptor membrane^{16,17}, which seems to be of great functional importance¹⁸: it secures the greatest possible absorption of incident light of all possible vibration planes by way of the membrane parallel orientation of the chromophore molecules and by the rotational motion of rhodopsin.

The question can be raised whether the hydrophilic oligosaccharide chain of rhodopsin has a definite form and arrangement with respect to the plane of the membrane, indicating its possible contributory role in the maintenance of the order of rhodopsin in the disk membrane. So far no information about the steric arrangement of the oligosaccharide chain of rhodopsin has been available.

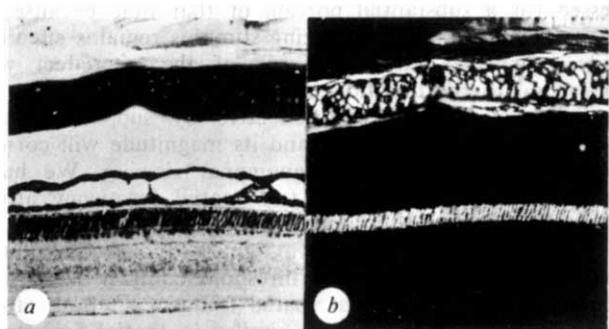


Fig. 2 After depigmentation and preoxidation with 0.1% HIO₄ (30 min) the slice was kept in aqueous saturated sodium bisulphite (30 min) and then stained with toluidine blue at pH ~1.0, stabilised with potassium ferricyanide. *a*, At this pH the cell nuclei remained unstained but there is strong basophilia of the outer segments of the retinal rods, sharply delineated against the unstained inner segments. (The basophilic granules—with weak mosaic-like birefringence in *b*)—seen around the cell nuclei in the scleral row of the outer nuclear layer were glycogen granules and could be removed by diastase.) At the top the basophilic scleral cartilage is seen; The same area as in, (*a*) with the polaroids crossed. The basophilic outer segments reveal a strong toluidine blue-induced negative birefringence with respect to their lengths, with retardations of about 30 nm at 600 nm. The cartilage shows strong toluidine blue induced birefringence²⁰.

Since we have developed a polarisation optical method for demonstrating linearly ordered glycol groups in polysaccharide chains, we have used this method in an attempt to decide whether the oligosaccharide chains of rhodopsin have a definite orientation with respect to the plane of the disk membrane. The method is based on the preoxidation by periodic acid of the vicinal OH groups, or amino-alcohol groups, of carbohydrates to dialdehyde groups, which after addition of sodium bisulphite, become negatively charged and able to bind toluidine blue at pH 1.0. This results in strong basophilia of the reacting compounds as reported for glycogen¹⁹ and, as we have found, in a strong toluidine blue induced birefringence: negative in character with respect to the oligosaccharide chains when they are present in a linear order (as in neutral mucins, basement membranes and starch granules). As all the carbohydrate residues of the oligosaccharide chain of rhodopsin (three glycosamine, two mannose and one galactose) may be expected to react with periodate²⁰ as confirmed by the strong periodic acid Schiff reaction of the rod outer segments, the method proved promising for the investigation of whether or not the carbohydrate chain of

rhodopsin was arranged in a definite order with respect to the plane of the disk membrane.

Dark-adapted retinae of the frog (*Rana esculenta*) were fixed by perfusion from the aorta in 10% formaldehyde solution, in a mixture of formaldehyde (5%), and glutaraldehyde (2%), and in Zenker fixative. Good polarisation optical results were obtained with formaldehyde and Zenker. Glutaraldehyde, a bifunctional cross-linking agent markedly depressed the optical effect, but not the induced basophilia, apparently because of the extensive cross linking of the underlying structure, which inhibits oriented dye binding. Paraffin slices were investigated in the light and polarisation microscopes. Data on the periodic acid bisulphite addition (PA-BSA) reaction and toluidine blue staining are included in the legend of Fig. 1. In Fig. 1*a*, the rod outer segments do not stain with toluidine blue at pH 3.0 and are isotropic in the mounting medium used (Fig. 1*b*). After PA-BSA reaction and toluidine blue staining (Fig. 2*a*) the outer segments reveal strong basophilia sharply delineated against the inner segments and corresponding to the staining with the PAS reaction, both indicating the presence of vicinal OH groups in great density in them. After the PAS reaction, however, the outer segments are optically isotropic, which means that this histochemical reaction of the vicinal OH groups gives no information about the molecular structural order of the underlying carbohydrate component in the photoreceptor membranes. On the other hand after PA-BSA reaction and toluidine blue staining the basophilic outer segments appear strongly birefringent (Fig. 2*b*) with a dispersion curve of their birefringence as a function of the wave length of the light as shown in Fig. 3. In it a diagram is included to show the underlying molecular mechanism of the PA-BSA reaction and the oriented toluidine blue binding of the carbohydrate chain with the resulting anisotropy.

It is obvious that optical anisotropy as a result of oriented molecular textures²¹. Therefore, the toluidine blue-induced anisotropy of the outer segments after PA-BSA reaction cannot be explained as arising either on randomly

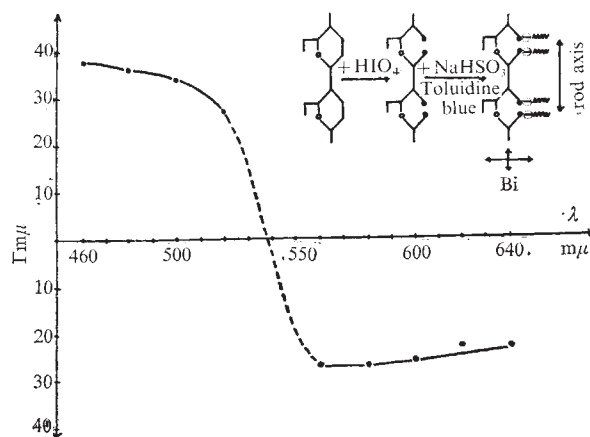


Fig. 3 Dispersion curve of the toluidine blue-induced birefringence of the rod outer segments of Fig. 2*b*, which are negatively birefringent for light of wave length over λ 540 nm and positive under λ 530 nm. In the zone of immersion (-----) no exact measurement of birefringence was possible. The inversion of birefringence is the result of anomalous dispersion of the refractive indices of toluidine blue when present in an oriented system²¹. As a result of the dispersion of the toluidine blue induced birefringence anomalous colours were seen: blue at additive, and red at subtractive compensation. The negative birefringence for the long-wavelength side of the spectrum indicates membrane-parallel orientation of the dye molecules²¹ and in turn, membrane perpendicular orientation of the reacting oligosaccharide chains of rhodopsin.

arranged straight oligosaccharide chains or on irregularly banded oligosaccharide chains of the rhodopsin molecules. Thus, from these polarisation optical findings it may be concluded that the oligosaccharide chains of rhodopsin have a straight linear form oriented perpendicularly to the plane of the disk membrane.

It seems reasonable to assume that these straight hydrophilic carbohydrate chains of the rhodopsin molecules protrude into the aqueous area of the disk membranes. In this way they may form an axis for each rhodopsin molecule, which may well act as an axis for their rotational motion as inferred from the polarisation optical studies of Brown¹³ and Cone¹⁴, and suggested by Vanderkooi²³ in his membrane model. It is interesting to note that Heller², who was the first to demonstrate the oligosaccharide chain as an integral part of rhodopsin, suggested that by hydrophilic interactions the carbohydrate moiety probably served as an orientation factor for the rhodopsin molecules in the membrane. Corless²⁴ even assumed that the carbohydrate chains might serve as rotation axes for the rhodopsin molecules, although there had been no evidence for a definite orientation of these side chains with respect to the plane of the disk membrane. Our polarisation optical observation indicating a straight form of the oligosaccharide chains of rhodopsin with perpendicular orientation to the plane of the disk membrane, strongly supports such a view on the structural-functional significance of this part of the rhodopsin molecule.

GY ROMHANYI
LENKE MOLNAR

Department of Pathology,
University of Medicine H-7643 Pécs,
Szigeti út 12, Hungary

Received February 5, 1974

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Adaptation to invisible gratings and the site of binocular rivalry suppression

THE visual system can be described usefully as a hierarchical series of functional stages, the initial stage being physical impingement of the stimulus and the terminal stage being visual perception. Psychophysical investigations typically provide rigorous specification of the first and last stages in the sequence, but a more complete explanation clearly must include information about intermediate stages as well. At present, however, very few techniques are available for analysing inferentially the intermediate stages¹. By incorpor-

ating two separate phenomena, binocular rivalry and visual aftereffects, into one paradigm, we describe here another technique for inferential analysis of the stages leading to vision. This technique indicates the sequence of stages underlying both phenomena.

Binocular rivalry refers to the transitory, phenomenal suppression of each eye's view produced when the two eyes receive different stimulation². Even though a rivalry-inducing stimulus continuously impinges on the retina, it is only intermittently visible. Several characteristics of rivalry indicate that it is a reflexive process, the suppression state resembling in many ways a central scotoma³⁻⁷. Visual aftereffects refer to temporary perceptual distortions produced by prolonged exposure to a stimulus, some familiar examples being the waterfall illusion⁸, figural aftereffects⁹, and the more recently discovered McCollough effect¹⁰. Up to a limit, the magnitude of most aftereffects increases with duration of exposure. This property of aftereffects, coupled with the unique dissociation of physical stimulation and phenomenal viewing characteristic of rivalry, provides the keystone to the technique reported here. Imagine that an aftereffect-inducing stimulus impinges on an eye for a certain duration, but at the same time is phenomenally suppressed for a substantial portion of that time because of binocular rivalry. If the inducing stimulus remains effective while suppressed, the magnitude of the aftereffect will correspond to the duration of physical stimulation. But if the stimulus is rendered ineffective by suppression the aftereffect will be weakened, and its magnitude will correspond to the duration of phenomenal viewing. We have applied this technique using two spatial frequency aftereffects, both of which are induced by prolonged exposure to a grating pattern of repetitive light and dark bars. One aftereffect is an elevation in threshold contrast¹¹, and the other is a shift in apparent spatial frequency¹². Both aftereffects are limited to gratings similar in spatial frequency and orientation to the adapting grating. We have found that suppression exerts no influence on the growth of either of these aftereffects, and take this as evidence that the stage where suppression occurs is located subsequent to the site of these aftereffects.

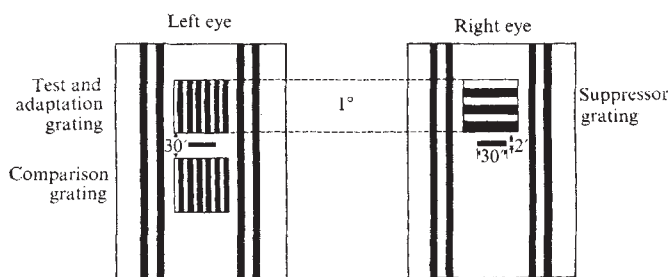


Fig. 1 The haploscopic stimulus arrangement used to induce and measure the spatial frequency after effects. Spatial frequency (number of cycles of the grating per degree of visual angle) and contrast (difference between the maximum and minimum intensities in the grating divided by their sum) could be altered, and the gratings turned off or on, without disturbing the mean luminance, 17 cd m⁻².

The displays used in our experiments are illustrated in Fig. 1. Squarewave gratings, masked to 1° square by trans-illuminated plastic sheets, were generated on cathode-ray tubes¹³ mounted onto the arms of a large haploscope. Subjects viewed the displays from a distance of 91.6 cm through 3 mm artificial pupils, with head movements minimised by a bite-board and headrest. Space-average luminance of each grating was 17 cd m⁻², independent of spatial frequency or contrast, and the luminance of the masks was 5.1 cd m⁻². Each grating could be turned off or on without changing the average luminance. With the two displays

fused, the horizontal suppressor grating appeared in binocular coincidence with the upper left-eye grating, with a small fixation bar appearing immediately below. Thin vertical stripes on each side of the gratings facilitated fusion of the two displays.

We used a matching procedure to measure the after-effects. While staring at the fixation bar, subjects quickly adjusted the test grating, using a potentiometer, to match the comparison grating on the basis of either contrast (experiment 1) or spatial frequency (experiment 2), offsetting the potentiometer after each adjustment. During these adjustments the right-eye suppressor grating was turned off. Adjustments were made before and immediately after exposing the left eye to an adaptation grating which temporarily replaced the test grating. During this adaptation period the subject gazed back and forth along the fixation bar to discourage formation of an afterimage. Each experiment consisted of four conditions of adaptation: (a) condition T—the adaptation grating was continuously present and visible for t s, with the right-eye suppressor grating turned off; (b) condition 2T—the adaptation grating was continuously present and visible for $2t$ s, with the suppressor grating off; (c) condition BR—the adaptation grating was physically present for $2t$ s but was seen for a total of no more than t s due to rivalry suppression produced by the suppressor grating presented to the right eye; and (d) condition RM—the adaptation grating was physically present and visible for t s distributed intermittently over $2t$ s, intermittency being produced by alternately exposing the left-eye adaptation grating and the right-eye suppressor grating following a pattern mimicking the fluctuations of rivalry. Each trial thus involved a preadaptation match of the test grating to the comparison grating, exposure to one of four adaptation conditions, followed immediately by a postadaptation match of the test grating (which fell on adapted retina) to the comparison grating (which fell on unadapted retina). Seven trials were devoted to each adaptation condition, the order of trials being haphazard. On each trial of condition BR, subjects continuously reported on rivalry fluctuations using a switch connected to an event recorder. Subjects rested at least 5 min between trials, until the aftereffect had dissipated.

Before an experiment, two adaptation durations, different by a factor of 2, were selected for each subject to maximise differences in the magnitude of the aftereffect under study. Only durations of 60 s or less were selected, for beyond this period the magnitude of the aftereffects no longer increased. Also, the spatial frequency and contrast of the adapting grating were adjusted for each subject to insure at least 50% suppression of the adaptation grating. Of three subjects, two were naive regarding the purpose of the experiments.

In experiment 1, the comparison grating was set for each subject at threshold contrast, and the spatial frequencies of the test, comparison, and adaptation gratings were identical. For two subjects the left-eye gratings were 3 cycles per degree, while for the remaining subject they were 6 cycles per degree. For each subject the contrast of the adaptation grating was approximately 1 log unit above threshold. With these spatial frequency and contrast values, the adaptation grating was suppressed for at least 50% of the adaptation period on each trial of condition BR, as determined from the graphic records of rivalry. The duration of adaptation t (condition T) varied from 15 to 30 s among subjects. The results from this first experiment are summarised in the graphs of Fig. 2a, which show for each subject the magnitude of the elevation in threshold contrast produced by each of the four conditions of adaptation. While all conditions produced some adaptation, the aftereffect for condition BR was significantly greater ($P < 0.01$, t test) than either condition T or RM and was at least as large as that for condition 2T. Control trials were included to determine (i) if the

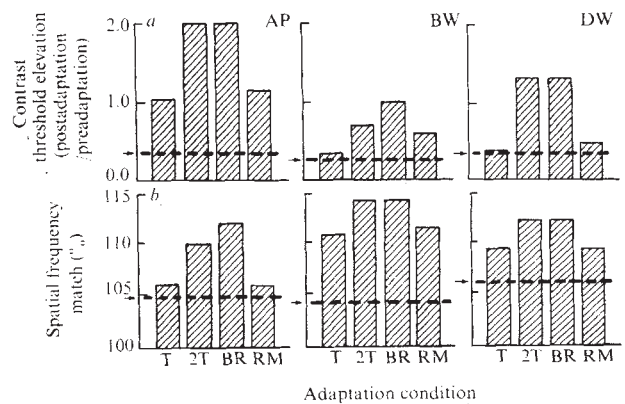


Fig. 2 The three bar graphs in (a), one for each subject, show the magnitude of the elevation in threshold contrast for four conditions of adaptation. Each value represents the mean ($n=7$) ratio of the adapted threshold to the unadapted threshold, minus 1.0. No aftereffect is thus represented by a value of 0.0. The arrow and dashed horizontal line indicate for each subject the level of significant threshold elevation (a ratio value equivalent to $2\sqrt{2}$ times the standard error of the unadapted thresholds). The average standard error for adapted thresholds was only about 0.03 log units. The three bar graphs in (b) show the magnitude of the shift in apparent spatial frequency, expressed as the mean ($n=7$) percentage difference from unadapted frequency matches. No aftereffect would yield a value of 100. Again the arrow and dashed lines mark the level of significant aftereffect, based on unadapted standard errors.

suppressor grating alone influenced the visibility of the test grating, (ii) if the effects of the adaptation grating spread to the comparison grating, and (iii) if the aftereffect was limited to frequencies similar to that of the adaptation grating. Results confirmed that the aftereffect was frequency specific and unconfounded by either the suppressor grating or by interaction between the adaptation and comparison gratings. Considered together, this pattern of results demonstrates that suppression exerts no influence on the growth of the threshold elevation aftereffect.

In experiment 2, in which we studied the size aftereffect¹², the spatial frequency of the comparison grating was 6 cycles per degree and the adaptation grating was 8 cycles per degree; all gratings were 1 log unit above threshold contrast and the durations t varied from 15 to 30 s among subjects. Results from this experiment, shown in Fig. 2b, follow the same pattern of results and significance found in the previous experiment. Because this aftereffect involves a change only in spatial frequency, not in contrast, these results extend the generality of those obtained in the first experiment.

In both instances the magnitude of the aftereffect was determined solely by the duration of physical impingement, phenomenal suppression exerting absolutely no influence. Yet from other data we know that suppression exerts potent inhibitory effects, at both the psychophysical³⁻⁵ and electrophysiological level^{6,7}. Thus the failure of suppression to weaken either aftereffect must mean that spatial frequency information arrives at the locus of the aftereffects even during suppression. This in turn implies that the neural mechanism responsible for suppression resides within the visual system after the site of the aftereffects which is generally presumed to be visual cortex^{11,12,14}. Our conclusion is based, of course, on the quite plausible assumption that pattern information is processed in serial fashion from eye to cortex.

Our experiments have helped to clarify the interaction between pattern adaptation and rivalry suppression. The technique of functionally uncoupling stages of the visual system by means of suppression, however, seems generally

applicable to other phenomena involving time-dependent effects and suppressible stimuli.

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RANDOLPH BLAKE*
ROBERT FOX

Department of Psychology,
Vanderbilt University,
Nashville, Tennessee 37240

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* Present address: Sensory Sciences Center, University of Texas Graduate School of Biomedical Sciences, Texas Medical Center, Houston, Texas 77025.

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Memory in enzyme membranes

THE problem of information storage in the phase of 'short term memory', has attracted considerable attention. A plausible mechanism for the process is based on an all-or-none process, assuming the existence of metastable states^{1,2}. Such phase transitions were thought to require structural changes in macromolecular components or in the membrane permeability of the information storage unit³⁻⁵.

The rapid development in the techniques of manufacturing synthetic enzyme membranes⁶ provides a new tool for demonstrating the existence of hysteretic phenomena which do not occur as a result of structural changes in a system.

The idea is based on studies by chemical engineers in which cooperative effects resulting from a nonlinear temperature dependence of rate coefficients were investigated from the point of view of the stability of chemical reactors. The existence of multiple stationary states was demonstrated for a reaction mixture having different reaction rates for the chemically controlled region and the diffusion-controlled region under the same external parameters. As a result the possibility of hysteresis and oscillations in internal parameters of the systems when coupled with thermal flows arose and was discussed⁷.

In analogy to these results, we demonstrate here the existence of hysteretic phenomena, through simple kinetic mechanisms which are shared by most enzymes. We refer to the two simple cases; autocatalysis by the product, and inhibition by excess substrate. Both are examples of systems which do not satisfy the Le Chatelier-Braun principle, and the phenomenon never takes place along the whole range of concentration values, thus resulting in a maximum in the activity. When coupled with diffusion of the product of substrate respectively, there is, because of the nonlinearities in the reaction rate, the possibility of three stationary states, of which two are stable and one unstable, for a limited range of parameter values⁸.

Two enzymatic reactions were studied. The hydrolysis of benzoyl arginine ethyl ester (BAEE) by papain (EC3.4.4.10) and the oxidation of uric acid by uricase (EC1.7.3.3). In

both systems displaced far from equilibrium (namely, high pH for the papain system (of which the product is a strong acid, pH=3.38) or excess of substrate for the uricase system) an autocatalytic effect of the product, or substrate inhibition, respectively is seen. When the reagents are subject to diffusion limitation (for example when bound to a membrane matrix), the description 'stationary state' implies equal rates of supply and consumption through the diffusional flows and the chemical processes, at any point in the reaction space. The rate of hydrolysis is a function of BAEE concentration, hydrogen ion concentration and their diffusivities, and the rate of oxidation is a function of uric acid concentration, oxygen concentration, and their diffusivities. Under zero order kinetics for the oxygen or BAEE, a degree of freedom is lost, thus eliminating the occurrence of oscillatory behaviour, demonstrated previously^{9,10}, and forcing the systems to remain in one of the two stable stationary states, depending on its history.

Two different methods were used in these studies. The papain membrane was produced on the surface of a rotating pH glass electrode, by pouring a solution of collodion (3%, w/v), in ether (48%), ethanol (50%) and water (2%), on the surface of the electrode bulb (Metrohm, 'combined', while rotating horizontally around its revolution axis at 100 r.p.m. After 2-3 min, the membrane-electrode was introduced into

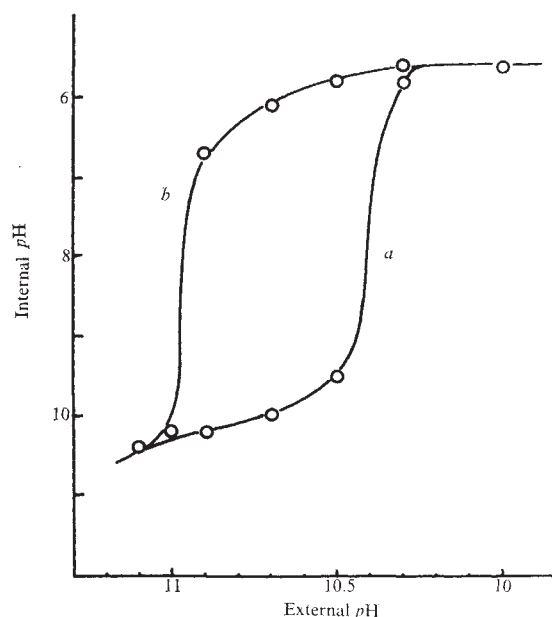


Fig. 1 Papain membrane-coated electrode. Stationary state description of internal pH as a function of increasing (a), and decreasing (b), the external pH values. The experiment started with decreasing the pH and then increasing. (See text for details.)

a solution of nonactivated papain (1 mg ml⁻¹), 0.1 M KCl for 3 d in 4° C (ref. 11), and then introduced to a solution of glutaraldehyde (0.5%) in phosphate buffer (0.02 M, pH 6.8), for 3 h at room temperature. The immobilised enzyme was activated by immersion in a solution containing EDTA (1 mg ml⁻¹), cysteine (1 mg ml⁻¹) and 0.1 M KCl, and then introduced in water, at 60° C, for 90 min, to cause partial denaturation, and to reduce the activity. It was then reintroduced to the cysteine EDTA solution, substrate was added and the pH at the electrode-membrane interface was recorded as a function of time and external pH. The results are given in Fig. 1, showing a hysteresis loop with two steep transitions, from pH 9.7-5.8 and from pH 6.7-10.2. Each point describes a quasistationary state of the system under constant external pH, controlled by a pH-stat appa-

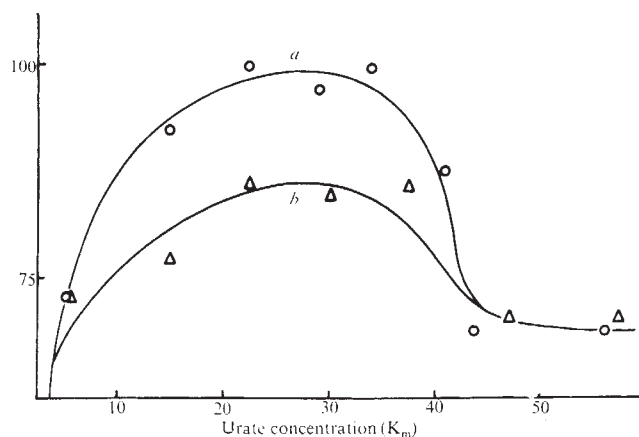


Fig. 2 Uricase membrane immersed in a solution of substrate. Enzyme activity (IU) as a function of the substrate concentration in the bulk solution. Results are given for increasing (a) and decreasing (b) substrate concentration value. (See text for details.)

ratus adding NaOH. At each point, an additional amount of substrate was added, verifying zero order kinetics. Because of the high rate of BAEE hydrolysis catalysed by hydroxyl ions at pH values higher than 10.5, compared with the low reaction rate, excess substrate was also verified at the end of the experiment. This was done by reducing the pH of the solution to 7, removing the electrode, adding papain to the solution and monitoring the amount of base added. The external pH was first lowered and then raised to eliminate an apparent effect due to possible loss of activity.

The uricase membrane was produced on a plane glass surface. A cross-linking reaction occurred for 2 h in a solution containing 30 mg ml⁻¹ albumin, 2 mg ml⁻¹ glutaraldehyde and 50 mg ml⁻¹ uric acid in phosphate buffer 0.02 M, pH 6.8. Before spreading on the glass surface, 0.5 mg ml⁻¹ of uricase was added to the solution¹². To stir and to reduce the mass transfer problems of oxygen, the measurements were performed with several pieces from the same membrane. The pieces were immersed in a buffered solution of substrate (glycine-NaOH buffer, pH 3.3, 0.05 M) and put in continuous air bubbling. A time gradient of urate is established by a controlled pumping (chemical stat) and the rate of substrate consumption was recorded spectrophotometrically at 293 nm. Though the oxygen concentration in the system is much lower than the urate concentration, zero order kinetics for oxygen exists because the oxidation of urate to allantoin is the rate limiting step in the reaction chain¹³.

The kinetic behaviour of both enzymes, in solution, was extensively studied^{14,15} and no metastable states were found. It is clear that the memory which had been introduced into the above systems by diffusion limitations is more probable than a memory mechanism based on molecular hysteresis. It demonstrates once again the importance of taking structural influence into account, especially when dealing with complex biological systems bearing enzymes.

A. NAPARSTEK

Polymer Department,
The Weizmann Institute of Science,
Rehovot, Israel

J. L. ROMETTE
J. P. KERNEVEZ
D. THOMAS

ERA No. 338 du CNRS,
Université de Technologie de Compiègne,
60 Compiègne, France

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Correlation between speed of pollen tube growth and seedling height in *Zea mays* L.

EVIDENCE of a correlation between gametophytic and sporophytic qualities could be useful because gametophytic competition is both frequent and severe in natural populations¹. A statistically significant correlation has been found in *Zea mays* between the relative speeds with which pollen tubes penetrate the lengths of styles and the relative competitive abilities of the resultant zygotes². This correlation was found in a study of mixtures of two pollen types when the sporophytic competition was limited to interactions between zygotes developing within a common inflorescence. It is therefore possible that faster growing pollen tubes allow a relatively early start in postfertilisation growth which accounts for the competitive superiority of the resultant zygotes, although there is a conflicting report³.

The present study was designed to test for correlations between relative speed of pollen tube growth and relative seedling weight, avoiding the possible influence of differences in time of fertilisation. Pollen types, of known tube growth rates, were each applied to separate inflorescences on plants of known genotypes. Five replications of each cross were made. All genotypes used were highly inbred and none of the crosses was a self pollination. The relative speeds of pollen tube growth were determined by the ceratation technique⁴, modified to allow quantitative expression of the results².

From each of the 35 well-filled ears produced by the unmixed pollinations of the present study, 36 seeds were removed from the middle segment, avoiding the extreme sizes of apical or basal seeds. Seeds were weighed individually and planted singly in sand-filled pots 2 inches square in size. The environment was controlled at 22±1° C, 2,500 f.c., and daylength 18 h. Fourteen days after planting, seedlings were cut at ground level and weighed.

The data were subjected to partial correlation analysis with three variables: the relative speed of faster growing tubes², relative seedling weight and relative seed weight (see legend to Table 1). Partial correlation analysis tests for correlations between any two factors and, in effect,

Table 1 Crosses made and the results observed.

Pair No.	Inbred lines used and their genotypes*	Number of well-filled ears obtained	Relative speed of faster pollen	Relative seedling weight† from faster pollen	Relative seed weight‡ from faster pollen
1	BB = yy × 16 = YY	2	0.0017	0.928	0.988
	BB = yy × 8 = yy§	2			
2	3A = yy × 1c = yy	1	0.0057	1.088	0.993
	3A = yy × 6A = YY§	3			
3	BB = yy × 1c = yy	3	0.0088	0.938	0.988
	BB = yy × 6A = YY§	2			
4	BB = yy × 16 = YY	2	0.0154	1.209	0.988
	BB = yy × 3B = yy§	3			
5	BB = yy × 16 = YY	2	0.0108	0.996	0.978
	BB = yy × BB = yy§	2			
6	BB = aa × 9c = aa	1	0.0198	1.183	1.049
	BB = aa × BB = AA§	3			
7	BB = aa × 9A = aa	2	0.0300	1.697	1.008
	BB = aa × BB = AA§	3			
8	16 = YYaa × 12 = AA	2	0.0281	1.148	1.021
	16 = YYaa × 9 = 1aa§	2			

*Lines designated as BB were obtained from Dr Robert Briggs, now of Funk Seed International, Inc., Bloomington, Illinois. All others were supplied by the Maize Genetics Cooperative.

†Average weight of seedlings resulting from fertilisations by gametes of the faster growing tubes divided by that from fertilisations by gametes of the slower growing tubes.

‡Average weight of seeds from fertilisations by gametes of faster growing tubes divided by that from fertilisations by gametes of the slower growing tubes.

§Faster pollen of the pair.

removes the influence of additional factors. Thus, in the present study, the value $r_{12.3}$ refers to the correlation between the first two factors, relative pollen tube speed and relative seedling weight, when the influence of the third factor is removed (Table 2).

The partial correlation between relative pollen tube speed and relative seed weight, $r_{12.3} = 0.566$, is not statistically significant, nor is that between relative seedling weight and relative seed weight ($r_{23.1} = -0.241$). The latter result does not indicate that relative seedling weight and relative seed weight are generally independent of each other, it just means that in this study, no statistically significant portion of the variation in one factor could be attributed to variation in the other factor. This is understandable in that a separate analysis of variance (not presented here) indicated that if each inflorescence receives

pollen from only one plant, weights of seeds resulting from fertilisations by gametes of the relatively fast pollen tubes do not differ significantly from weights of seeds produced by gametes of relatively slow pollen tubes, a conclusion in agreement with that of Jones¹.

The partial correlation coefficient between the first factor, relative pollen tube speed, and the second, relative seedling weight, with the influence of the third, relative seed weight, removed is $r_{12.3} = 0.761$, which is significant at the 5% level (d.f. = 5 with two variables) (ref. 5). This demonstrates that a significant number of genetic factors which are expressed in the gametophyte are also expressed in the sporophyte.

This statistically significant correlation between relative pollen tube speed and relative weight of the resultant seedlings cannot be due to differences in time of fertilisation because there were no interactions between the two types of zygotes. It also suggests that seedling vigour, a selectively important quality⁶, can be influenced by gametophytic competition.

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DAVID L. MULCAHY

Table 2 Correlation and partial correlation analyses for relative pollen tube speed, relative seedling weight, and relative seed weight.

r_{12}	= 0.781*
r_{13}	= 0.608
r_{23}	= 0.355
$r_{12.3}$	= 0.761*
$r_{13.2}$	= 0.566
$r_{23.1}$	= -0.241

*Significant at the 5% level.

r_{12} , correlation coefficient between the first and second factors.

$r_{12.3}$, partial correlation coefficient between the first two factors with the influence of the third removed.

Department of Botany,
University of Massachusetts,
Amherst, Massachusetts 01002

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Heterocyst formation in a blue-green alga, *Cylindrospermum*

THE blue-green alga *Cylindrospermum*, unlike a similar alga, *Anabaena*, where intercalary heterocysts are present, contains only two terminal heterocysts (Fig. 1A). When either or both the heterocysts are detached, new heterocysts regenerate in time. In *Anabaena cylindrica*, where the heterocysts occur at regular intervals, the spacing pattern is determined by intercellular interactions, probably involving some inhibitory substance produced by the heterocyst itself. No two heterocysts are produced side by side either in *Anabaena* or *Cylindrospermum*. The inhibitory substance could be ammonia or a substance derived from ammonia¹. Here we report observations which indicate that an inhibitor controls the spacing of heterocysts.

Clonal cultures of *Cylindrospermum trichosporum* were grown on Allen and Arnon's medium², both on solid and liquid media. In some experiments it was also cultivated on media containing combined nitrogen, ammonium chloride (3 mg ml⁻¹) or ammonium phosphate (3 mg ml⁻¹). The growth and formation of heterocysts was followed by the methods described by Kale *et al.*³. Algal material, generally 15 d old, was suspended in fresh medium and blended in a modified kenmix blender for 0.5–1 min and after dilution, drops of the suspension were placed on agar plates. Selected filaments were focused under the microscope and observations made.

The number of vegetative cells present between the two terminal heterocysts is a fixed characteristic of an algal population for a particular set of culture conditions in *Cylindrospermum*, the average length of the filament between the two heterocysts is 418 μ m (107 cells). On liquid media with constant shaking, filaments tend to be longer than the average and while on agar, the spreading filaments

heterocyst and initial length of 42.8 μ m, increased to 142.8 μ m (2.38 times), and another with an initial length of 100 μ m grew to 157 μ m (1.75 times) before the second heterocyst developed. The interheterocyst distance in 52 cases (total 100) was 141–150 μ m and in 74 cases 131–160 μ m, with an average value of 145 μ m as the minimum length needed for the regeneration of the second heterocyst.

When fragments with no heterocysts attached to them were plated on agar plates they invariably regenerated one heterocyst first before further growth and the formation of the second heterocyst. The amount of growth preceding the formation of first heterocyst is variable and is not always related to their initial length but seems to depend largely on their location on the intact filament from which they were derived. The second heterocyst, however, generally formed when the average length of the fragment attained a range of 141–150 μ m.

When the alga was grown on combined nitrogen, ammonium chloride or ammonium phosphate, the filaments were free of heterocysts because, as in *Anabaena*, combined

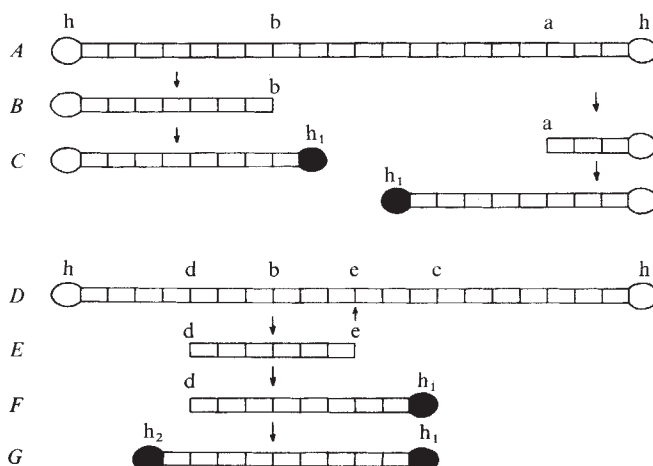


Fig. 1 Diagrammatic representation of a filament of *Cylindrospermum* showing the position of heterocysts (h) and the regeneration of new heterocysts (h_1 , h_2) in the fragments. A–G, explanation in text.

Table 1 Growth needed before the formation of first heterocyst in fragments of *Cylindrospermum* grown on various media.

Control (Allen and Arnon's medium)	36*	54†
Medium with ammonium chloride, 3 mg ml ⁻¹	55*	41†
Medium with ammonium phosphate, 3 mg ml ⁻¹	51*	18†

* % increase in length.

† No. of observations.

break into two, usually each fragment having a single heterocyst, generally 111–150 μ m (21–28 cells) long. When the filaments are broken into fragments by blending at high speed, the fragments so formed also have a single heterocyst and are of a similar range in length (111–130 μ m). This seems to indicate the presence of naturally occurring weak points of breakage in the filaments of *Cylindrospermum*.

Fragments of filaments with one heterocyst still attached, produced by natural breaking or by high speed blending, regenerate the missing heterocyst at the broken end when placed on agar (Fig. 1B and C). This is always preceded by growth, however, which is found to be inversely related to the initial length of the fragment. A fragment with single

nitrogen inhibited their formation. If the filament fragments were taken from such cultures, the first heterocyst appeared in them after a prolonged growth on nitrogen-free medium, more than what was needed for a fragment previously grown on medium free of combined nitrogen (Table 1).

If heterocysts do produce an inhibitor that controls the formation of other heterocysts, its concentration in a vegetative cell would be proportional to its distance from the existing heterocyst. Accordingly, the end of the longer fragment which is farther from the heterocyst develops another heterocyst more quickly (after growth) than the one in a shorter fragment. The fragment must grow before the formation of a second heterocyst to reduce the level of the inhibitor.

Heterocyst formation in fragments which do not already contain them (Fig. 1E), is very different, however. Such fragments can arise from any portion of the intact filament (Fig. 1D), either from the middle portions or eccentrically from one side (Fig. 1D, b–c, d–e). The fragment b–c may contain the inhibitor at equal concentrations at both ends and when heterocysts develop they may arise simultaneously after a limited growth. On the other hand, in the fragment d–e, the inhibitor level would be greater at d than at e (as d is nearer to a heterocyst) and as a result the fragment

develops a heterocyst first at e and later at d, only when growth has taken place to reduce the inhibitor to a sufficiently low level.

During our work, we found very few cases where the heterocysts formed simultaneously in a fragment. In general, it seems that in *Cylindrospermum*, filaments do not easily break from the middle region and that almost all fragments arise eccentrically on either side of a mid point between the two terminal heterocysts. The successive formation of heterocysts in a fragment containing no heterocysts is also an indication of the existence of polarity in the filament of the alga, which is itself a reflection of a gradient in the level of the inhibitor. A normal filament may have a decreasing gradient of the inhibitor starting from the terminal heterocyst to the mid point of the filament, the other half of the filament being a mirror image of this.

When fragments of *Cylindrospermum* were taken from the alga previously grown in a medium containing combined nitrogen and plated on agar medium free of ammonia, they were found to regenerate their first heterocyst only after a prolonged time, during which greater growth has taken place in them compared with the fragments grown earlier on molecular nitrogen. Presumably, by growing on combined nitrogen, the internal level of the inhibitor remained at a high level and as growth took place decreasing its concentration, the formation of heterocysts was initiated. The heterocysts appeared sequentially, however, first at one end and later at the other. This shows that the filaments whether grown on molecular nitrogen or combined nitrogen, still show polarity, that is their terminals are predetermined with regard to regeneration.

The nature of the inhibitor produced by heterocysts to control their spacing pattern is still debatable¹⁻⁵ but recent findings indicate that heterocysts are the site of nitrogen fixation⁵. Fogg's suggestion^{6,7} that ammonia concentration in the filament is responsible for such an inhibition gains further ground, but it may not be the only deciding factor¹.

We have already discussed in some detail⁸ how the spacing of heterocysts could be controlled in *Anabaena* in terms of the known metabolic activities of the heterocysts as well as the vegetative cells (see also, Wahal *et al.*⁹ for the role of ascorbic acid). Although filament polarity is found in many blue-green algae, such as *Rivularia*, nothing is known of its nature and maintenance. In *Anabaena*, it has been shown¹⁰ that cell division is asymmetrical and heterocyst spacing is controlled by the interaction between developing cells¹¹ but it is yet to be seen whether a similar situation exists in *Cylindrospermum* also.

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P. M. REDDY
E. R. S. TALPASAYI

Laboratory of Algal Physiology,
Department of Botany,
Banaras Hindu University,
Varanasi - 221005, India

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GENERAL

Commercial radionuclide dispensing

COMMERCIAL suppliers of radionuclides operate under stringent regulations. Most companies give specific assurances of nuclide identity and purity. Examples are the plots of γ spectra, radiochromatograms and, most important, the data sheet that specifies such things as quality, purity and radiation intensity of the specific nuclide. Unfortunately, however, the controls used by suppliers are inadequate and the technical data sheet cannot be relied upon implicitly.

In support of other reports¹⁻³ I shall relate an experience which involved a shipment of radioiron. On arrival, the package was monitored and the γ activity was about 10% of that expected. The supplier confirmed this and explained that a technician had calculated the stock to be 28.7 mCi ml⁻¹ instead of 2.87 mCi and ml⁻¹ had therefore dispensed only 10% of the required volume. Despite the small volume, however, the radiation value show, on the technical data sheet was that expected for 2 mCi of ⁵⁹Fe (about 70 mr h⁻¹), which suggested that the number was either recalled from memory or copied from an old sheet. I stated this in a letter and the company responded with a written acknowledgment of error. The compounded errors, they explained, were because of rapid expansion and shortage of personnel. They believed that safeguards and routine controls, (which were not specified), would be effective in the future.

Eliminating the monitoring step and entering an assumed value for radiation were serious errors. Companies should enforce separate duties, so that a person who dispenses radionuclides cannot also perform assays, complete data sheets, package, monitor and authorise final release for shipment.

It seems reasonable, as a partial answer to this problem, that if readout plots of γ spectra or chromatograms of various nuclides can be supplied to customers, then automatic recordings of radiation could also be provided. Such records could be inspected by supervisors to detect nuclide deficiency or excess and then be duplicated to provide a customer copy. The production of an authentic radiation report for the sample would be of the greatest importance. This should help eliminate the temptation to take short cuts and to falsify reports with assumed radiation values.

LEE O. TIFFIN

Agricultural Environmental Quality Institute,
United States Department of Agriculture,
Agricultural Research Service,
Beltsville, Maryland 20705

Received March 29, 1974.

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book reviews

Lobotomy

Psychophysiology of the Frontal Lobes. Edited by K. H. Pribram and A. R. Luria. Pp. xii+332. (Academic: London, August, 1973.) \$19.95.

THE starting point of interest in the frontal lobes was the celebrated "Crowbar Case" reported by Harlow in 1848. One Phineas Gage, a quarry worker, had a large iron tamping rod blown by a stick of dynamite into his jaw and and out through the top of his head, producing serious injury to both frontal lobes. As a result, Gage underwent a disastrous change of personality. From having been an efficient and capable foreman, he became fitful, irreverent and obstinate, and is said to have "indulged in profanity that would have embarrassed an 18th century British Sea Captain". Thus was born the "frontal lobe syndrome", the study of which in modern times owes much to the Russian neuropsychologist Alexander Luria, himself a coeditor of this volume.

Although a subject of fascination to psychiatrists and others, the frontal lobes have hitherto excited comparatively little interest among physiologists, largely because they are in the main electrically inexcitable and their excision does not occasion gross defects in sensation or movement. Indeed it was not until systematic behaviour experiments were begun in the thirties that any appreciable progress was made. These experiments claimed to reveal some fairly constant behaviour deficits in subhuman primates following bilateral frontal excisions and also—incidentally—led Moniz to inaugurate the era of psychosurgery. Dr Karl Pribram, likewise a coeditor, has been active over many years in charting the behavioural deficits produced in monkeys by circumscribed cerebral excisions. Their underlying physiology, however, has remained largely unexplored.

This volume represents an attempt, if a somewhat diffuse and ill-coordinated one, to fill this gap. Based upon a symposium on frontal lobe function held during the 18th International Congress of Psychology in Moscow some years ago, it brings together fourteen papers concerned with the neurophysiology of the frontal lobes and the effects of frontal lesions on the electrical activity of the human brain. Of the contributors, fourteen are American (mostly based on the West Coast), eight are Russian (all based on Mos-

cow) and one is English (based on Bristol). The history of the enterprise and its relation to other recent symposia on closely related topics is outlined in a brief preface.

Taken as a whole, this volume reveals a distinct difference in approach as between the Russian and American contributors, though there is, of course, a good deal of common ground. By and large, the Russian work is almost exclusively limited to experiments with human subjects, mainly evoked potential and EEG studies, whereas the Americans give far more attention to the data of animal experiments and to explanation of behavioural deficits in physiological terms. Although much of the work is pretty specialised, it does serve to give an overview of current physiological work on the frontal lobes, though alas there is nowhere any real sign of a breakthrough.

In his concluding chapter, Pribram discusses frontal lobe functions in terms of spatial and temporal organisation and, more interestingly, the inhibitory mechanisms that, when deranged, produce the distractibility so marked in the 'frontal' animal or patient. He also places stress on the frontal cortex as the "executive" of the brain in the overall control of behaviour. In this latter claim, at least, he and Luria see eye to eye.

O. L. ZANGWILL

Breaking bones

Mechanical Properties of Bone. By F. Gaynor Evans. Pp. xiii+322. (Thomas: Springfield, Illinois, June 1973.) \$25.75.

THIS interesting and well illustrated compendium of data on the mechanical properties of bone is a necessary reference work for all biologists and engineers who are concerned with the mechanical properties of materials. For the biologist, and this includes the orthopaedic surgeon, neurosurgeon, orthodontist and others who must be particularly interested in the effects of force either applied to, or generated within the body, an understanding of the mechanical properties of bone, which forms some 18% of the body, is essential for the prevention and treatment of a variety of conditions afflicting the musculo-skeletal system. An assessment of the change in mechanical properties of biopsy specimens of bone in the course of disease and its treatment may before long become a diagnostic and prognostic procedure.

For the engineer an appreciation of

the design of the skeleton, the structure of bone and its mechanical properties is of considerable interest. In particular the materials scientists and structural engineers who are concerned with the design of passenger-carrying vehicles, which are increasingly involved in accidents, need the information concerning the mechanical properties of the living item contained within the vehicle. This book deals with, in the main, the mechanical properties of bone as a material. The thoughts of those who are responsible for the development of new materials may well be stimulated by the section dealing with the effects of the microscopic structure of bone when considered as a structural material.

For those who are interested in the mechanical properties of the whole bone this work should be read in conjunction with the author's other publication *Stress and Strain in Bones*.

JOHN T. SCALES

Water crossing membranes

Water Transport in Cells and Tissues. By C. R. House. Pp. vi+562. (Monographs of the Physiological Society, No. 24 Arnold: London, January 17 1974) £10.50.

THIS book is a mine of information and the fact that more than 900 references are included in the bibliography is a measure of the thoroughness with which the relevant literature has been surveyed. Of the total of ten chapters, seven make unexceptionable reading and the accounts of transcapillary and transepithelial water transport are outstandingly clear and comprehensive. The permeability characteristics of cell membranes in general and of artificial membranes also receive detailed and informative consideration as do the physical and chemical properties of water, which are dealt with at the beginning of the book.

The account of fluid dynamics in the embryo is less impressive, although this seems to be due more to the shortage of precise information than to any fault of the author, who is led to the conclusion that "our ignorance of the processes that trigger, enhance and finally block the formation of extra-embryonic cavities is enormous". One may question the wisdom of devoting nearly 25 pages to the exposition of this topic, except perhaps as a challenge to future investigators, to whom the message could have been given rather more

succinctly. On the other hand, the treatment of water movement in plants is somewhat skimmed and includes virtually no discussion of transport in the xylem. I was particularly disappointed to find no reference to the extraordinary ability of the giant Redwood trees to raise water.

The author in his preface refers to the dilemma which faced him in deciding upon the place of mathematical theory in his book, a dilemma which he resolved by introducing a short "practical preamble" at the beginning of his chapter entitled "Phenomenological Description of the Transport Process", and then following this with a fairly lengthy excursion into irreversible thermodynamics. For the *cognoscente* this excursion is probably unnecessary; the presentation is too compressed to be really helpful to the novice and does not bring out at all clearly the thinking behind the development of irreversible thermodynamics from its more familiar progenitor, equilibrium thermodynamics, sometimes dubbed "thermostatics". These are contrasted in terms of entropy changes rather than of the notion of moving from states to rates. But the mathematics used is relatively straightforward and furthermore, the succeeding parts of the book can be read profitably without any particular mathematical expertise.

Dr House's approach to his subject is commendably judicial and the evidence for and against the ideas which he discusses is carefully evaluated and, where appropriate, he has interposed a 'summing up'. Occasionally he abandons his detachment and confesses, with engaging candour, to personal bias. For example, this bias is freely admitted following a spirited and critical examination of the widely accepted dogma that active transport of water is inadmissible on energetic grounds. A plausible, if slightly confusing case, based as it is on a mixture of reversible and irreversible thermodynamics, is put forward for bringing in a verdict of "not proven" on this highly controversial issue.

This monograph forms a worthy addition to the series published for the Physiological Society. To predict that it will successfully stand comparison with some of the best of its predecessors implies both an established criterion for judgement and at the same time a deserved compliment to Dr House.

R. V. COXON

Warm blooded

Temperature Regulation in Mammals and other Vertebrates. By John Bligh. Pp. xix+436. (North-Holland: Amsterdam and London; American Elsevier, New York, 1973.) \$30.20.

DR BLIGH is to be congratulated on writing a book which is stimulating and concise, covering the whole field of thermoregulation in vertebrates—an area in which he himself is a distinguished worker. The book is written from the physiological standpoint and it will prove unsatisfying to physiological ecologists, since, for instance, many of the adaptations shown by mammals living in unusual environments are mentioned only briefly or not at all. It includes chapters on the traditional areas of thermoregulation (central control, sensing systems, effector mechanisms, neuronal influences, central transmitter substances and so on).

There are very stimulating chapters on the various models of the thermoregulation system which have been proposed, and there are useful sections on unorthodox theories, fever and exercise. In the section on comparative thermoregulation there seems to be no mention of the recent work on thermoregulation in certain large fish. This, however, does not take away from the overall high standard of the book which is highly recommended. It is a well produced book but perhaps rather expensive.

J. N. R. GRAINGER

Scientific literature

Understanding Scientific Literature: a Bibliometric Approach. By Joseph C. Donohue. Pp. xiii+101. (MIT: Cambridge, Mass. and London, 1973.) \$10.

TWENTY years ago, the American librarian Jesse Shera was advocating the study of a 'social epistemology' defined as "the analysis of the production, distribution and utilisation of intellectual products in much the same fashion as that in which the production, distribution and utilisation of material products have long been investigated". He pointed out that all the requisite data are to be found in the books and journals that fill the shelves of our libraries. By analogy with econometrics the term bibliometrics was later coined to cover the essentially data-collecting and analytical phase of a more theoretical study, which would be concerned with the organisation of mankind's exosomatic stores of knowledge for more effective social use.

In an uncoordinated way the statistical analyses Shera called for have been developed in recent years and the slim volume under review is the first monograph wholly devoted to these matters. One would expect the author of such a work to offer a critical review and constructive coordination of the widely scattered contributions and thus present the first consolidation of the emergent study. The need for such a work is now more urgent than it was twenty years ago because during that time large-scale computerised documentary information systems have been developed and the feasibility of even larger world systems is now being discussed. But all systems so far designed have been based on the very questionable assumptions that all published scientific papers have equal scientific weight, that 100% coverage is a desirable goal and that all papers retain their scientific interest *sine die*. These systems can only become more effective, more frequently used and less costly when their designers pay attention to the use made of scientific information by its scientific users—a use which is fully documented within the scientific journals themselves.

There are several techniques which bear on these matters and which one would expect to find described in a 10-dollar monograph. Two empirical laws have been known for some time. In 1925, Lotka noted that the number of authors who contribute r papers to any specified subject literature over any specified period is proportional to $1/r^2$ (though the exponent is 1.89 rather than 2). And in 1934, Bradford noted that, when bibliographical sources are ranked in order of decreasing productivity, the number of items contributed

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by the source of rank r is proportional to $1/r$ if r is greater than a certain limit—an empirical law which applies to many other social contexts. More recently, Goffman has shown that the dissemination of new ideas is closely analogous to the spread of infectious diseases with endemic outbreaks preceding the take-off to epidemic growth. He has considered the conditions that have to be satisfied before take-off can occur and also the conditions under which scientific communication begins, to break down under the weight of its own documentation.

It has also been noted that scientific literatures not only grow at different exponential rates but decay exponentially at comparable rates, with half-lives of four to 16 years, so that the 'information explosion' worries only those who wish to keep everything—alive or dead. Work has also been done, mainly in the United States, on 'bibliographic coupling'—a technique of tracing who cites whom to identify networks of scientific interaction. There is therefore a varied and comprehensive task to be done by the author of the first monograph describing these techniques and their applications to information system economy.

Unfortunately, a great opportunity has been missed. The author makes a personal selection from the techniques. He inadequately and uncritically discusses only the Bradford law, the epidemiological model and some cross-citation techniques. He makes idiosyncratic selections of illustrative material drawn wholly from American sources and bibliographically ill defined. The numerical conclusions and graphs are firmly expressed though they are based on modest samples with no intimation that sampling errors might arise. The 100-page monograph reduces to 70 pages of text and 30 pages of singularly uninformative graphs. Thus the price of this modest introductory essay of 20,000 words is highly inflationary.

B. C. BROOKES

Neutron generators

Activation Analysis with Neutron Generators. By Sam S. Nargolwalla and Edwin P. Przybylowicz. Pp.xvii+662. (Chemical Analysis: A Series of Monographs on Analytical Chemistry and Its Applications.) (Wiley Interscience: New York and London, January 1974.) £16.25.

It is often very important to be able to take one's microanalytical kit with one around the Universe. This has not been easy with neutron activation analysis, since fission reactors are heavy and one does not take them easily down a mine or to the moons of Jupiter. But small generators of fusion

neutrons (say using the D-T reaction) can be made, although they have some problems such as the stability of their neutron yield.

This book attempts to summarise the uses of generators and can be described as "nearly a handbook" on the subject. A handbook normally leads you through mazes of research material easily, smoothing out the little wrinkles in the patchwork of data. In parts of this book, I feel the authors have indeed essayed such a task of inspection of pedigree and smoothing of data. In others, the reader is not sure whether a table of figures on, say, a type of neutron reaction is raw from one man's research or represents the sum of knowledge. The intent of many of these tabulations, which could have been properly signalled in only a few words, is sometimes left unclear. Thus, this book is perhaps best described as a research review which nearly achieves handbook status.

The authors certainly attempt completeness of coverage; they have used the prodigious documentation services of the US National Bureau of Standards (NBS) at which institution they once worked together, to compile the knowledge of the bureau and others on the use of neutron generators and the course of nuclear reactions activated by fusion neutrons.

The techniques for the generation of neutrons and the exposure of samples are lovingly described, with detailed engineering drawings and dosimetric maps. A section follows on the treatment of the diverse types of systematic error which can interfere with the analytical accuracy of the method and this section certainly convinces that, for certain important elements, neutron activation can surpass X-ray fluorescence or atomic absorption methods. It is, however, this first half of the book, which is somewhat littered with tables of doubtful intent, that will neither help the student nor satisfy the man in search of a handbook; by contrast, the second half of the book consists of a 300-page chapter that takes in turn each element which will succumb to activation analysis. The authors tabulate the nuclear reactions which work with fast neutrons, and then give for each a set of comments and data useful to the analyst, including a uniform set of computer-generated curves for buildup of radioisotope against time, decay against time and so on. Here, the reader's confidence in completeness and uniformity can be high. Finally, there are several appendices giving tabulations, by other workers, of those nuclear reactions which are useful in analysis. These are doubtless useful to have to hand but are, again, insufficiently clear in intent.

The impression left is that neutron activation analysis is a formidable subject to venture into unless armed with much knowledge and time. This is not the expressed intention of the authors, who wish to stimulate the broader use of the method and fairly state the advantages and disadvantages of the method compared with those of the rival techniques. They have certainly done a great service in dragging out and starting to organise the files of the NBS on this subject: however, it will take a firmer hand to encourage the hesitant to embark on this technique or to construct a true handbook out of all that information. On the other hand, a scientist already committed will learn all about the special techniques needed to maintain analytical accuracy and all the rest of the practical issues in this undoubtedly useful field of work.

A. G. HOLMES-SIEDLE

Stars for students

Introduction to Stellar Atmospheres and Interiors. By Eva Novotny. Pp.xii+543. (Oxford University: London and New York, September 1973.) £7.00.

THIS book is intended as a textbook for senior undergraduates and commencing graduate students. It is a generally well written and well produced book but in my opinion the balance of the book is wrong for a textbook. The basic concepts of the subject tend to be described very briefly and important fundamental results are often quoted without proof, whereas many pages of tables and diagrams are devoted to the detailed results of particular solutions of the equations of stellar structure and evolution.

Two examples will serve to illustrate what I mean. About fifteen pages are devoted to tables of the monochromatic absorption coefficient in stellar atmospheres and a further ten pages to tables of the properties of model stellar atmospheres, but there is no detailed discussion of the form of the equations which must be solved to obtain a model stellar atmosphere, except in the case of a grey atmosphere in local thermodynamic equilibrium. It is mentioned that there is a maximum mass for white dwarf stars and a reference is given to where a physical explanation of the existence of a maximum mass can be found. The book would have been much improved if one page of tables had been sacrificed so that a brief explanation of the existence of the maximum masses of cold bodies of various types and of the near inevitability of the existence of black holes could have been included.

One danger of including too many

Plant Cell Structure and Metabolism

J L Hall, T J Flowers and
R M Roberts.

An up-to-date treatment of plant metabolism in relation to cell structure. Current knowledge of the plant cell is frequently reviewed in the light of what is known about mammalian or microbial systems, particularly for the areas where plant science is not so far advanced.

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East African Vegetation

E M Lind and
M E S Morrison

This book describes the major types of vegetation to be found in Uganda, Kenya and Tanzania. These are dealt with under the main headings of forests, rangelands, inland aquatic vegetation, the vegetation of the sea coast, high mountain vegetation, the factors affecting vegetation, climate and soils, and a short history of the vegetation of the region.

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Longman
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detailed results in a first textbook is that they may be regarded as being more reliable than they really are. Although in some cases it is made clear that the results quoted only represent one point of view, this is not always true. In the discussion of the evolution of stars approaching the main sequence, the Hayashi theory is fully tabulated but no mention is made of the later work by Larson which casts considerable doubt on the previous results.

More detailed criticisms of the book include the feeling that far too much space is devoted to the Vogt Russell theorem which is certainly not generally true and that the concepts of thermodynamic equilibrium and local thermodynamic equilibrium should have been described much more clearly. It is also a pity that the discussion of numerical methods does not include the Henyey technique, which has been most widely used in recent years.

Now that I possess the book I may well find it very useful to have in one volume so many tables and figures illustrating recent studies of stellar structure and evolution and students will similarly be able to use it as a reference book. But it seems very unlikely that for students it will replace such texts as that by Schwarzschild, which is considerably cheaper and which gives a much deeper physical understanding of the subject.

R. J. TAYLER

Magnetic rocks

The Physical Principle of Rock Magnetism. By Frank D. Stacey and Subir K. Banerjee. Pp. ix+195. (Developments in Solid Earth Geophysics, Vol. 5.) (Elsevier: Amsterdam, London and New York, 1974.) Dfl.60; \$2.10.

THIS book was advertised for over a year before its appearance, but it has been worth waiting. It is a slim volume deliberately planned as a physical supplement to Irving's *Palaeomagnetism and its Application to Geological and Geophysical Problems* (Wiley, 1964). It must also be considered to effectively replace Nagata's excellent, but outdated book, *Rock Magnetism* (Maruzen, 1961) although it does not contain any instrumental discussion that formed a significant part of Nagata's work.

The organisation is logical, starting with the basic magnetic properties of solids and then turning to the common ferromagnetic minerals, magnetite and haematite and their solid solutions with ilmenite and ulvöspinel. This chapter certainly brings the reader up to date on the current view of the changes in parameters for synthetic solid solutions and pure minerals, but more informa-

tion on the effects of impurities would have been useful. Only the two pages on pyrrhotite are not really adequate for a mineral that is extremely complex and not all that rare. Single and multi-domain theories are considered next, first in relation to grains of magnetite and then haematite. The outline and discussion tends to follow closely Stacey's ideas, originally published in 1963, as does the subsequent treatment of the effects of time and temperature.

The next chapter is devoted to the remanence of sediments and is followed by a discussion of chemical remanence that is largely confined to exsolution behaviour as titanomagnetite solid solutions are oxidised. The effect of anhysteretic magnetisation and stress fields then receive considerable, warranted attention. The latter is, however, concerned almost entirely with stress effects on the direction of remanence and does not consider the converse, that is using magnetic anisotropy as a means of determining strain and hence evaluating past stress fields. The evidence for reversals of the geomagnetic field and possible self-reversal mechanisms are considered in rather a generalised way; the correlation between petrology and polarity that has been established in some regions is dismissed as being "paradoxical" in only four lines.

Finally extraterrestrial magnetisation is discussed and the remanence of meteorites is attributed to strong solar fields when the Sun passed through a T-Tauri stage. Strangely no mention is made of the magnetic properties of lunar rocks—presumably to avoid being overtaken by more recent findings.

The book is well written, adequately illustrated and its production is up to the usual high Elsevier standard. The only significant weakness is really in terms of the magnetisation of sedimentary rocks which are discussed in less than 5% of the total book, yet more and more sedimentary rock types are now being investigated and the magnetic effects during cementation and the diagenetic changes of detrital grains are much more important than simple detrital magnetisation. A little more on rotational hysteresis and physical techniques for identifying both the composition and physical parameters of ferromagnetic particles in rocks would have been beneficial. The bibliography is really a reference list that effectively only goes up to 1971 (only six references for 1972 with one "in press" and one "in preparation" for 1973). The authors defend their continued use of c.g.s. units, although the use of MKS can only come about when the textbooks adopt the system. These, however, are essentially quibbles about a book which must form part of any geophysics library.

D. H. TARLING

obituary

S. N. Bose

PROFESSOR SATYENDRA NATH BOSE, the eminent physicist and propounder of Bose-Einstein statistics died on February 4, 1974 just as the Golden Jubilee of Bose statistics was being celebrated. He was 80 years old.

S. N. Bose was born in North Calcutta on January 1, 1894 and his interest in mathematics and the classics was kindled early in his childhood. After passing the entrance examination from the Hindu School, he joined the Presidency College where he came into close contact with eminent teachers like Sir Jagadis Bose, FRS, Sir Prafulla Ray and the principal, H. M. Percival. He obtained his MSc in 1915 in mixed mathematics along with M. N. Saha. They were both appointed the next year as lecturers in physics in the newly founded University College of Science of Calcutta. His first research publication (jointly with M. N. Saha) was *On the Influence of Finite Volume of Molecules on the Equation of State* followed by two papers on *The Stress Equations of Equilibrium* and *On the Horpolhode* published in the Bulletin of the Calcutta Mathematical Society. In 1920 he published his work *On the Deduction of Rydberg's Law from the Quantum Theory of Spectral Emission*.

During this period, Calcutta University published a booklet on relativity which contained the earliest available English translation of Einstein's papers rendered by S. N. Bose and M. N. Saha with a preface by P. C. Mahalanabis. Bose joined the Dacca University as a Reader in Physics in 1921. His epoch-making paper on *Planck's*

Gesetz und Lichtquanten hypothese was published in *Zeit für Physik*, the translation into German being done by Professor Einstein himself. This paper contained the first correct treatment of the thermodynamics of the photon gas and laid the foundation of quantum statistics. His next paper, *Warmegleichgewicht im Strahlungsfeld bei anwesenheit von Materie* (*Zeit für Physik*), which he considered to be his best scientific contribution, was also translated and communicated by Einstein, but with a critical comment.

The same year he obtained two years' study leave from the Dacca University and went first to Paris where he spent some time in the laboratories of Madame Curie, Langevin, Maurice and Louis de Broglie. The following year he stayed in Berlin participating in seminars and discussions with celebrities like Planck, Schrödinger, Pauli, Heisenberg, Sommerfeld and Einstein. He also spent some time in the X-ray crystallography laboratory at Berlin-Dahlem with Polanyi.

On his return to India in 1927, he became a professor and the Head of the Department of Physics at Dacca University. In 1945 he came back to the University College of Science, Calcutta, as the Khaira Professor of Physics and held the Chairmanship of the department from 1950-56. He joined the Visva Bharati University as its Vice-Chancellor in 1956 and continued there until 1958. He was awarded a DSc (Honoris causa) by the Calcutta University in 1957 when it was celebrating its centenary. Later on, distinctions were showered on him by a number of universities and institutes.

In 1958 he was elected an FRS. He was the President of the National Institute of Sciences of India (1949-50) and also the General President of the Indian Science Congress Association (1944). He was a nominated member of the Rajya Sabha (the Upper House of Indian Parliament) for some time. In 1959 he became a National Professor attached to the Calcutta University and held the position until his death.

In the years following the publication of his famous paper of 1924, his research interests were many. They include his works in D^2 -statistics (1936), on total reflection of electromagnetic waves in the ionosphere (1938), Lorentz group (1936), on Dirac equation and Zeeman effect (1943) and on an integral equation formulation of the hydrogen atom problem (1945). His forte was a profound understanding of basic science, which found expression in branches other than physics and research workers from varied disciplines benefited from discussions with him. During the years 1952-54 he took renewed interest in the theory of relativity.

He also made significant contributions to Einstein's unified field theory which were published in *Comptes Rendus* (1953), *J. Phys. Radium Paris* (1953) and *Ann. Math.* (1954), respectively.

Professor Bose was the Founder President of the *Bangiya Bijnan Parishad*, and of the Bengali science journal *Jnan O Bijnan*. He was closely associated with the cultural renaissance in Bengal.

He leaves his wife, two sons and five daughters.

Announcements

International Meetings

June 25, **Sixth Form Conference on Science and Technology** (History and Social Science Subject Group, c/o The Admissions Office, University of Sussex, Falmer, Brighton BN1 9QH, UK).

August 4-8, **11th International Meeting of the Society for Cryobiology** (Dr D. E. Pegg, Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ, UK)

August 4-17, **NATO International**

Summer School on Genetic Manipulations with Plant Material (Professor L. Ledoux, Botany Department, Université de Liège, B 4000, Liège, Belgium)

August 5-9, **7th Semi-Annual Short Course on Laser Safety** (R. James Rockwell, Jr., Laser Safety Course, Conmed, 114 Medical College, Cincinnati, Ohio 45219).

August 5-24, **4th International Training Course on Membrane Biophysics** (Dr Peter F. Curran, Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510)

August 7-9, **23rd Annual Denver Conference on Applications of X-Ray Analysis** (Dr C. O. Ruud, Metallurgy and Materials Science Division, Denver Research Institute, University of Denver, Denver Colorado 80210)

August 7-15, **Honolulu Symposium on Population and the Family** (World Population Year Secretariat, UN Fund for Population Activities, 485 Lexington Avenue, New York, New York 10017)

August 7-15, **10th International Congress of Crystallography** (Tenth International Congress of Crystallography, P.O. Box 7205 Amsterdam, The Netherlands)

August 11-15, **International Youth Population Conference** (Secretariat, International Youth Population Conference, 5 chemin des Iris, 1216 Cointrin, Geneva, Switzerland)

August 11-24, **New Approaches to the Study of Fish Vision** (M. A. Ali, NATO ASI Director, Department de Biologie, Université de Montréal, Case Postale 6128, Montréal 101, Canada)

August 12-17, **16th International Ornithological Congress** (Secretary General, 16th International Ornithological Congress, P.O. Box 84, Lynnham, ACT, Australia 2602)

August 19-22, **4th Western Hemisphere Nutrition Congress** (Department of Foods and Nutrition, American Medical Association, 535 North Dearborn Street, Chicago, Illinois 60610)

August 19-22, **7th Annual Meeting of the Society for the Study of Reproduction** (Dr Hamish Robertson, Chairman, Local Committee SSR, Reproductive Research Section, Animal Research Institute, Ottawa K1A 0C6, Canada)

August 19-30, **World Population Conference** (Office of the Secretary General, World Population Conference, United Nations, New York, New York 10017)

August 22-29, **19th Congress of the International Association of Limnology** (Roscoe W. Dalke, Freshwater Institute, Department of the Environment, 501 University Crescent, Winnipeg, Manitoba, Canada R3T 2N6)

August 25-31, **2nd International Symposium on the Genetics of Industrial Microorganisms** (The Assistant Secretary (GIM 74), Society for Chemical Industry, 14 Belgrave Square, London SW1 8PS)

August 25-30, **9th Meeting of the Federation of European Biochemical Societies** (Secretariat of the 9th FEBS Meeting, H-1502 Budapest, P.O. Box 7, Hungary)

August 25-31, **8th International Congress on Electron Microscopy** (British Joint Committee for Microscopy, 74 Belgrave Square, London, SW1X 8QX)

August 26-30, **11th New Zealand National Electronics and Geophysics Convention** (Nelcon 74, Centre for Continuing Education, The University of Auckland, Private Bag, Auckland, New Zealand)

August 28-30, **International Symposium on Fertilisation in Higher Plants** (Professor H. F. Linskens, Department

of Botany, University Toernooiveld, Nijmegen, The Netherlands)

August 28-31, **International Meeting on Geochronology, Cosmochronology and Isotope Geology** (Dr J. Hamet, Laboratoire de Géochimie, et de Cosmochimie, Institut de Physique du Globe, 4 Place Jussieu 75005, France)

Reports and publications

Great Britain

Joint Services Expedition to North Peary Land, 1969. Pp. 111. (London: Lieutenant Colonel J. D. C. Peacock, REME, c/o Williams and Glyns Bank Ltd., Kirkland House, Whitehall, SW1A 2DL, 1974.) [181]

Water Resources Board. Water Resources in England and Wales. Vol. 1: Report. Pp. iv + 67 + 5 maps. £3.50 net. Vol. 2: Appendices. Pp. 56. £4.20 net. (London: HMSO, 1973.) [181]

Department of the Environment. Water Pollution Research 1972: Report of the Director of Water Pollution Research. Pp. v + 137 + 5 plates. (London: HMSO, 1973.) £1.10 net. [211]

Department of the Environment. Clean Air Today. Pp. vi + 36 + 7 plates. (London: HMSO, 1974.) £1 net. [221]

University of London. Consultative Committee for Co-ordinating Discussion on the Recommendations of the Murray Committee—First Report. Pp. 16. (London: University of London, 1973.) [231]

University Grants Committee. First Destination of University Graduates 1971-72. Pp. 78. (London: HMSO, 1973.) 85p net. [281]

The Hannah Research Institute. Report 1973. Pp. 61 + 4 plates. (Ayr, Scotland: The Hannah Research Institute, University of Glasgow, 1974.) [281]

Index of Reviews in Organic Chemistry, 1973 Supplement, Compiled by D. A. Lewis. Pp. 81. (London: The Chemical Society, 1973.) £1. [281]

Seventh Annual Report of the Institute of Development Studies at the University of Sussex, UK. Pp. 53. (Brighton: Institute of Development Studies, University of Sussex, 1974.) [281]

Royal Scottish Museum. Scottish Scientific Instrument-Makers, 1600-1900. By D. J. Bryden. (Information Series.) Pp. 59. (Edinburgh: Royal Scottish Museum, 1972.) [281]

Department of Education and Science. Public Libraries and Their Use. By John N. Taylor and Ian M. Johnson. (Library Information Series, No. 4.) Pp. vii + 82. (London: HMSO, 1973.) £1.10 net. [291]

The Zoological Record, 1970, Vol. 107, Section 2: Protozoa. Compiled by the Staff of the Zoological Society of London and R. A. Neal. Pp. vi + 276. £8.15. 1970, Vol. 107, Section 6: Vermes, Part A. Compiled by Eileen Mitchell and R. A. Bray. Pp. vi + 175. £6.65. (London: The Zoological Society of London, 1973.) [311]

Ministry of Agriculture, Fisheries and Food. Fisheries Radiobiological Laboratory. Technical Report FRL 9: Radioactivity in Surface and Coastal Waters of the British Isles, 1971. By N. T. Mitchell. Pp. 34. (Lowestoft, Suffolk: Fisheries Radiobiological Laboratory, 1973.) [311]

The Language Disordered Child: a New Look at Theory and Treatment. By G. N. Fraser and J. Blockley. Edited by P. A. Berse. Pp. 56. (Windsor, Berks: NFER Publishing Company, Ltd, 1973. Distributed in the USA by Humanities Press, Inc., New York.) £2.15. [311]

A Code of Practice for the Detailed Statement of Accuracy. By P. J. Campion, J. E. Burns and A. Williams. Pp. vii + 52. (National Physical Laboratory.) (London: HMSO, 1973.) £1.05 net. [311]

Other countries

Geological Survey of Western Australia. Annual Report for the Year 1972. Pp. 92. 1:250,000 Geological Series—Explanatory Notes. Charnley, Western Australia. Sheet SE/51-4 International Index. Compiled by D. C. Gellatly and R. Halligan. Pp. 35. Forrest, Western Australia. Sheet SH/52-10 International Index. Compiled by D. C. Lowry. Pp. 13. Geraldton, Western Australia. Sheet SH/50-1 International Index. Compiled by P. E. Playford, R. C. Horwitz, R. Peers and J. L. Baxter. Jubilee, Western Australia. Sheet SH/52-4 International Index. Compiled by D. C. Lowry. Pp. 12. Kurnalpi, Western Australia. Sheet SH/51-10 International Index. Compiled by I. R. Williams. Pp. 37. Lennard River, Western Australia. Sheet SH/58-8 International Index. Compiled by G. M. Derrick and P. E. Playford. Pp. 49. Menzies, Western Australia,

Sheet SH/51-8 International Index. Compiled by M. Kriewaldt. Pp. 11. Peak Hill, Western Australia. Sheet SG/60-8 International Index. Compiled by W. N. MacLeod. Pp. 21. (Perth: Geological Survey of Western Australia, 1970, 1971 and 1973.) [161]

Institute Royal des Sciences Naturelles de Belgique Mémoires. No. 171: L'Industrie du Site Paléolithique de Maisières-Canal. Par J. de Heinzelin. Pp. 62 + 45 planches. No. 173: Revision des Gastropoda du Danien et du Montien de la Belgique. 1: Les Gastropoda du Calcaire de Mons. Par Maxime Glibert. Pp. 116 + 11 planches. Résultats Scientifiques du Voyage aux Indes Orientales Néerlandaises de L. Aa. Rr. le Prince et la Princesse Léopold de Belgique. Table Analytique. Par Maxime Glibert. Pp. 48. Table Analytique des Mémoires de l'Institut Royal des Sciences Naturelles de Belgique. Mémoires 2^e Série, No. 145 à 159 (1959-1968). Mémoires 2^e Série, Nos. 58 à 83, (1959-1968). Par Maxime Glibert, Pp. 50. (Bruxelles: Institut Royal des Sciences Naturelles de Belgique, 1971 et 1973.) [171]

Chemistry in 1973. Pp. 67. (Washington, DC: American Chemical Society, 1973.) [171]

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Seed Science and Technology, Vol. 1, No. 1, 1973. (Proceedings of the International Seed Testing Association.) Pp. 1-280. (As, Norway: A. Wold, International Seed Testing Association, Box 68, 1973.) 150 Norwegian crowns (four numbers). [182]

Leukemia Society of America, Inc. Annual Report Fiscal Year 1973. Pp. 56. (New York, NY: Leukemia Society of America, Inc., 211 East 43rd Street, 1974.) [192]

World Health Organization. Technical Report Series, No. 535: Environmental and Health Monitoring in Occupational Health—Report of a WHO Expert Committee. Pp. 48. (Geneva: WHO; London: HMSO, 1973.) 5 Sw. francs; 75p; \$1.75. [192]

Publications du Centre National pour l'Exploitation des Océans (CNEXO). Série: Résultats des Campagnes à la Mer, No. 07-1974: Résultats de la Campagne Cinea-Charcot II (15 Mars 29 Avril 1971). (Groupe Mediprod). (Paris Cedex: Centre National pour l'Exploitation des Océans, 1974.) [192]

Population Bulletin, Vol. 29, No. 3: Hawaii—Growing Pains in Paradise. Pp. 40. (Washington, DC: Population Reference Bureau, Inc., 1973.) 50 cents. [192]

National Research Council of Canada. Annual Report on Scholarships and Grants in Aid of Research, 1972/1973. Pp. liv+566. (Ottawa: National Research Council of Canada, 1974.) \$2.50. [192]

Cell, Vol. 1, No. 1, January 1974. Edited by Benjamin Lewin. Published monthly. Pp. 1-64. Annual subscription: \$70 for individuals; \$100 for institutions. (Cambridge, Mass. and London: The MIT Press, 1974.) [192]

Publications de l'Institut National pour l'Étude Agronomique du Congo. Atlas Climatique du Bassin Congolais. Troisième Partie: Température et Humidité de l'Air, Rosée, Température du Sol. Par Franz Bultot. (Bruxelles: Institut National pour l'Étude Agronomique du Congo, 1972.) [192]

World Health Organization. Field Methods for the Control of Trachoma. Edited by M. L. Tarozzo. Pp. 48. (Geneva: WHO; London: HMSO, 1973.) 12 Sw. francs. [192]

Republic of Botswana. Annual Report of the Geological Survey and Mines Department for the year 1971. Pp. 34. 50 cents. Mineral Resources Report No. 3: Resources Inventory of Botswana—Industrial Rocks and Minerals. By N. W. D. Massey. Pp. vii+39. 2 rand. (Lobatsi, Botswana: Geological Survey Department, 1973.) [202]

United States Department of the Interior: Geological Survey. Water-Supply Paper 2034: Cost Analysis of Ground-Water Supplied in the North Atlantic Region, 1970. By D. J. Cederstrom. Pp. iv+48. 60 cents. Professional Paper 822: Water Resources of the Delmarva Peninsula. By E. M. Cushing, I. H. Kantowitz and K. R. Taylor. Pp. v+58 (plates 1-12). \$12.10. Professional Paper 808: Paleontology and Stratigraphy of the Rabbit Hill Limestone and Lone Mountain Dolomite of Central Nevada. By C. W. Merriam. Pp. v+50 + 12 plates. \$1.75. (Washington, DC: Government Printing Office, 1973.) [202]

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